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09273616	6261839	250	03/22/1999	METHOD FOR THE INDUCTION OF A NK CELL- MEDIATED IMMUNE RESPONSE	MULTHOFF, GABRIELE ✓
09646835	Not Issued	71	01/11/2001	Use of hsp70 proteins	MULTHOFF, GABRIELE
10380408	Not Issued	31	08/25/2003	Hsp70 peptide stimulating natural killer (nk) cell activity and uses thereof	MULTHOFF, GABRIELE ✓
10526586	Not Issued	19	01/01/0001	Use of granme b as an hsp70/Hsp70 peptide dependent inducer of apoptosis in tumor cells	MULTHOFF, GABRIELE

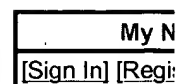
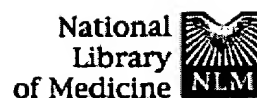
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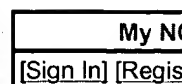
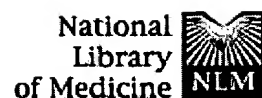
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☐ 1: Cancer Immunol Immunother. 1996 Dec;43(4):226-30. [Related Articles, Links](#)**Heat-shock protein 72 cell-surface expression on human lung carcinoma cells in associated with an increased sensitivity to lysis mediated by adherent natural killer cells.****Botzler C, Issels R, Multhoff G.**

GSF-National Research Centre for Environment and Health, Institute of Clinical Hematology, Munich, Germany.

The cell-surface expression patterns of major histocompatibility complex (MHC) class I, class II and heat-shock protein 72 (HSP72) molecules were measured on human lung (LX-1) and mammary (MX-1) carcinoma cells. No major differences were found in the MHC cell-surface expression pattern of both cell lines. However, they differ significantly in their capacity to express HSP72 on their cell surface. Under physiological conditions LX-1 cells express HSP72 molecules on more than 90% of the cells, whereas MX-1 cells exhibit no significant HSP72 cell-surface expression (less than 5%). These expression patterns remained stable in all further cell passages tested. The sensitivity to lysis mediated by an interleukin-2 (IL-2)-stimulated, adherent natural killer (NK) cell population could be correlated with the amount of cell-surface-expressed HSP72 molecules. By antibody-blocking studies, using HSP72-specific monoclonal antibody (mAb), a strong inhibition of lysis was only found with LX-1 cells but not with MX-1 cells. In contrast to the cell-surface expression, the cytoplasmic amount of HSP72 in MX-1 cells was twice as high compared to LX-1 cells under physiological conditions. After nonlethal heat-shock the rate of induction and the total cytoplasmic amounts of HSP72 were comparable in both cell lines. The clonogenic cell viability of LX-1 cells after incubation at temperatures ranging from 41 degrees C to 44 degrees C was significantly elevated compared to that of MX-1 cells. In conclusion we state the following: (i) HSP72 cell-surface expression on human carcinoma cells is independent of the cytoplasmic amount of HSP72; (ii) the cell-surface expression of HSP72 is associated with an increased sensitivity of tumor cells to lysis mediated by an IL-2-stimulated, adherent NK cell population; (iii) thermoresistance is not related to the cytoplasmic HSP72 level but

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ORIGINAL ARTICLE

Claus Botzler · Rolf Issels · Gabriele Multhoff

Heat-shock protein 72 cell-surface expression on human lung carcinoma cells is associated with an increased sensitivity to lysis mediated by adherent natural killer cells

Received: 20 June 1996 / Accepted: 25 September 1996

Abstract The cell-surface expression patterns of major histocompatibility complex (MHC) class I, class II and heat-shock protein 72 (HSP72) molecules were measured on human lung (LX-1) and mammary (MX-1) carcinoma cells. No major differences were found in the MHC cell-surface expression pattern of both cell lines. However, they differ significantly in their capacity to express HSP72 on their cell surface. Under physiological conditions LX-1 cells express HSP72 molecules on more than 90% of the cells, whereas MX-1 cells exhibit no significant HSP72 cell-surface expression (less than 5%). These expression patterns remained stable in all further cell passages tested. The sensitivity to lysis mediated by an interleukin-2 (IL-2)-stimulated, adherent natural killer (NK) cell population could be correlated with the amount of cell-surface-expressed HSP72 molecules. By antibody-blocking studies, using HSP72-specific monoclonal antibody (mAb), a strong inhibition of lysis was only found with LX-1 cells but not with MX-1 cells. In contrast to the cell-surface expression, the cytoplasmic amount of HSP72 in MX-1 cells was twice as high compared to LX-1 cells under physiological conditions. After nonlethal heat-shock the rate of induction and the total cytoplasmic amounts of HSP72 were comparable in both cell lines. The clonogenic cell viability of LX-1 cells after incubation at temperatures ranging from 41 °C to 44 °C was significantly elevated compared to that of MX-1 cells. In conclusion we state the following: (i) HSP72 cell-surface expression on human carcinoma cells is independent of the cytoplasmic amount of HSP72; (ii) the cell-surface expression of HSP72 is associated with an increased sensitivity of tumour cells to lysis mediated by an IL-2-stimulated, adherent NK cell population; (iii) thermoresis-

tance is not related to the cytoplasmic HSP72 level but might be related to the amount of HSP72 expressed on the cell surface.

Key words Carcinoma · Heat-shock protein 72 (HSP72) · Adherent NK cells · Immune response · Thermoresistance

Introduction

Intracellularly, heat-shock proteins (HSP) perform a variety of chaperoning functions. Under physiological conditions, these proteins bind to and stabilize non-native conformations of other proteins and therefore inhibit aggregation of unfolded proteins or enable translocation across membranes [2]. Heat-shock proteins fulfil essential functions in protecting cells against lethal damage induced by thermal or chemical stress. It has been reported that HSP70 differentially localizes to the nucleus, to the cytosol and to the cell surface [15, 16, 23]. Within the last few years evidence has been accumulating that HSP also play major roles in the antitumour immune response. Members of the HSP70 group have been found to be expressed on the cell surface of certain tumour cells either under physiological conditions [3, 4, 17, 20] or after heat stress [13, 14, 22], where they can act as immunogenic determinants for different effector cell types. In order to demonstrate cell-surface localization of HSP72, the major heat-inducible form of members of the HSP70 family, on different human tumour cell types, we used a highly specific monoclonal antibody (mAb), which was described by Welch [23]. As previously shown [13], this antibody is not crossreactive with the constitutively expressed HSP73, which shows more than 85% homology to HSP72 [7, 23].

In this report the intracellular and membrane-bound HSP72 expressions of two independent human carcinoma cell types under physiological conditions and after heat stress were compared and related to the immunogenicity and thermoresistance of the tumour cells.

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Materials and methods

Cell culture

The lung carcinoma cell line LX-1 (Tumorzentrums Heidelberg, TZB no. 610012, Germany) and the mammary carcinoma cell line MX-1 (Tumorzentrums Heidelberg, TZB no. 810023, Germany), were cultured in RPMI-1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Eggenstein, Germany) and antibiotics. Heat treatment was performed in a temperature-controlled water bath at temperatures ranging from 41.8 °C to 44 °C. Clonogenic survival of LX-1 and MX-1 tumour cells was determined by dose/response survival curves of LX-1 and MX-1 cells heated for the indicated lengths of time. The results are summarized from at least four independent experiments in which each data point represents four to six dedicated flasks. The cell viability was calculated with correction for plating efficiency.

Monoclonal antibodies (mAb), indirect immunofluorescence and fluorescence-activated cell sorting (FACScan) analysis

The antibodies W6/32 (IgG2a; MHC class I), L243 (IgG2a; HLA DR; kindly provided by Dr J. Johnson, München, Germany), HSP72 (IgG1; Amersham, Braunschweig, Germany), isotype-matched control antibodies (IgG1 and IgG2a, Dianova, Hamburg, Germany), anti-CD3 (IgG1, Dianova, Hamburg, Germany), anti-CD3/CD16+56 (IgG1/IgG1, Becton Dickinson, Heidelberg, Germany) and fluorescein-isothiocyanate-labelled rabbit anti-mouse IgG (Dako, Hamburg, Germany) as a secondary antibody were used for phenotypic characterization of tumour and effector cells on a FACScan instrument (Becton Dickinson and Co., Heidelberg, Germany) and for antibody-blocking studies of tumour cells. Briefly, viable cells (1×10^6) were incubated with the primary antibody for 30 min at 4 °C. After washing and incubation with the secondary antibody (30 min, 4 °C) the cells were analysed by FACS.

Sodium dodecylsulphate/polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis and quantification

After SDS-PAGE using a standard protocol of Laemmli [8] the proteins were transferred to nitrocellulose membranes [18]. After Western blot analysis the protein amount of HSP72 was detected with the electrochemiluminescence system (Amersham, Braunschweig, Germany) and quantified by laser densitometry.

Generation of adherent interleukin-2 (IL-2)-stimulated effector cells

Monocyte-depleted peripheral blood lymphocytes were separated into a CD3⁺ T lymphocyte fraction and a CD3⁻, adherent natural killer (NK) cell fraction by sequential incubation (three times) on plastic tissue-culture flasks in recombinant IL-2 medium (6000 IU/ml) for 24 h according to a protocol described by Vujanovic et al. [21]. After rejection of the non-adherent CD3⁺ lymphocyte population, the adherent cell population was cultivated in low-dose recombinant IL-2 medium (100 IU/ml) for 3 days and then used in the cytotoxicity assay. The phenotypic characterization of the adherent effector cell population (FACScan analysis as described above) revealed that these cells lack the ability to express CD3 (less than 6% of the cells express CD3) but express CD16+CD56 on about 30%–50% of the cells.

Cytotoxicity assay

The specificity of IL-2-activated (100 IU/ml), adherent NK effector cell population was monitored in a standard 4-h ^{51}Cr release assay [10]. LX-1 and MX-1 tumour cell lines were used as target cells. The effector to target (E:T) ratios ranged from 40:1 to 5:1. The percentage

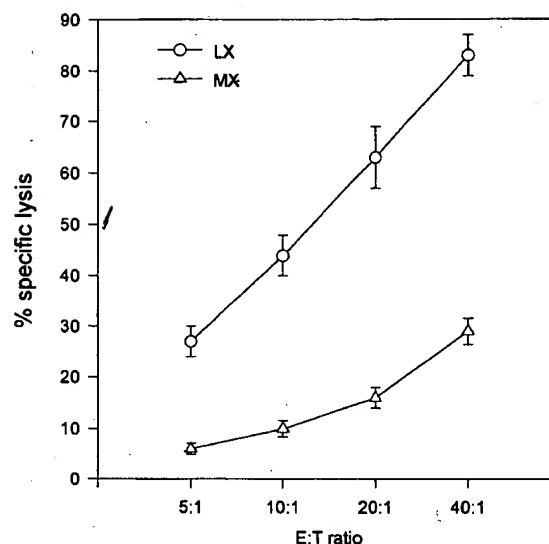


Fig. 1 Comparison of the sensitivity of LX-1 and MX-1 carcinoma cells to lysis mediated by interleukin-2 (IL-2)-stimulated, adherent natural killer (NK) effector cells (CD3: 3%–6%; CD16+CD56: 40%–50%). The effector-to-target ratio ranged from 40:1 to 5:1; spontaneous release (%) was below 20% for each target cell line. Each data point represents the mean value of at least four independent experiments; SD values are indicated as vertical bars

specific lysis was calculated as $[(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$. The percentage spontaneous release was calculated as $(\text{spontaneous release}) / (\text{maximal release}) \times 100$ and was always below 20% for each target cell.

The antibody-inhibition assays were performed by preincubation of the target cells with the anti-HSP72-specific mAb or with an isotype-matched control antibody at a final concentration of $5 \mu\text{g}/1 \times 10^6$ cells.

Results

At comparable cell densities LX-1 and MX-1 cells did not differ in their capacity to express major histocompatibility complex (MHC) antigens. Flow-cytometric analysis revealed that about 99% of LX-1 and MX-1 carcinoma cells express MHC class I molecules equally; no MHC class II expression was detected (less than 5% expression) on either of the carcinoma cell lines. However, HSP72 molecules were only expressed on the cell surface of LX-1 cells and not on MX-1 cells. These characteristics remained stable for all further cell passages. Determination of the plating efficiency, as a growth parameter, revealed no major differences between the two tumour cell types (0.94 ± 0.1 in LX-1 compared to 1.00 ± 0.25 in MX-1 cells). In an attempt to study the role of HSP72 cell-surface expression with respect to the sensitivity of these tumour cells to lysis by IL-2-stimulated, adherent NK cells, cytotoxicity assays were performed. As shown in Fig. 1 the LX-1 carcinoma cells were about 4.5-fold more sensitive to lysis than were MX-1 carcinoma cells. By antibody-blocking assays the sensitivity to lysis mediated by IL-2-stimulated, adherent

Fig. 2a, b Antibody blocking assay using IL-2-stimulated, adherent NK effector cells (CD3: 1%–4%; CD16+CD56: 30%–50%), HSP72 mAb and IgG1 isotype-matched control antibody. **a** The lysis of HSP72-expressing LX-1 carcinoma cells was inhibited by HSP72 mAb but not with an isotype-matched control antibody. **b** No inhibition of lysis was observed with MX-1 carcinoma cells nor with the HSP72-specific mAb nor with the isotype control antibody. The effector-to-target (E:T) ratio ranged from 40:1 to 5:1; spontaneous release (%) was below 20% for each target cell line. Each data point represents the mean value of at least three independent experiments; SD values are indicated as vertical bars

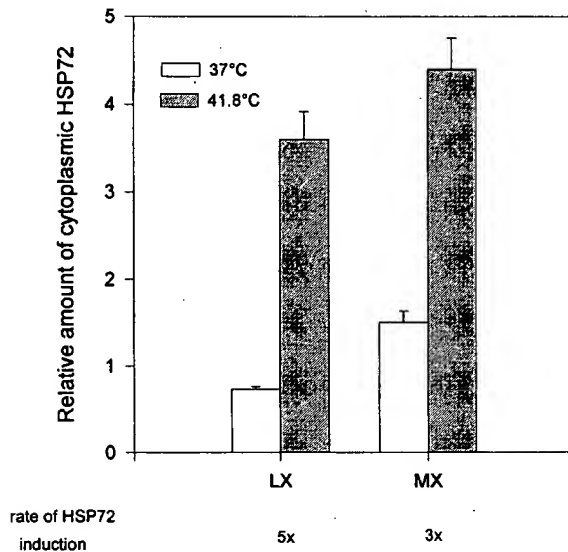
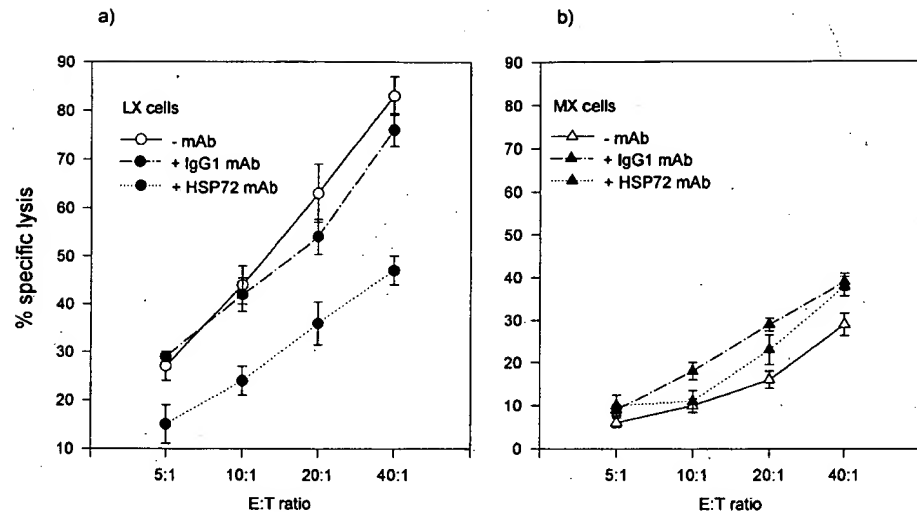


Fig. 3 Cytoplasmic amounts of HSP72 in LX-1 and MX-1 carcinoma cells under physiological conditions (37 °C) and after a mild heat-shock at 41.8 °C for 2 h (followed by a recovery period at 37 °C for 2 h). Quantification of the rate of induction of cytoplasmic HSP72 after heat-shock is shown below the graph

NK cells could be positively correlated with the amount of HSP72 that is expressed at the cell surface (Fig. 2): a strong inhibition of lysis was found when the HSP72-specific mAb on LX-1 cells that exhibit a high expression rate of HSP72 at the cell surface (more than 90%) was used, as demonstrated in Fig. 2a. No inhibition of lysis was detectable with the MX-1 cell line that lacks HSP72 expression on the cell surface (Fig. 2b). An IgG1 isotype-matched control antibody (Figs. 2a, 2b) and an MHC-class-I-specific antibody (data not shown) had no inhibitory effect on the lysis pattern of either of the two carcinoma cell lines LX-1 and MX-1.

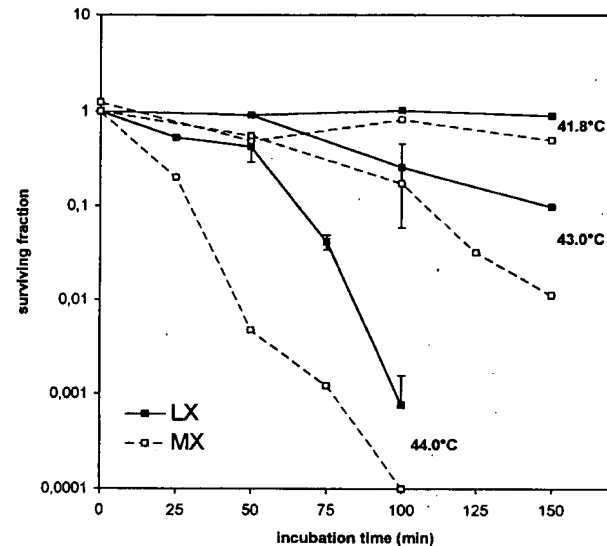


Fig. 4 Effects of in vitro heat treatment on lung (LX-1) and mammary (MX-1) carcinoma cells. Dose/response survival curves for incubations at 41.8 °C, 43 °C and 44 °C for the indicated time intervals ranging from 25 min to 150 min. Each data point represents four to six dedicated flasks; vertical bars represent SD values. The clonogenic cell survival of LX-1 carcinoma cells after heat treatment at the indicated temperatures was significantly higher than that of MX-1 carcinoma cells

As shown in Fig. 3 the cytoplasmic amount of HSP72 in MX-1 cells that exhibit no HSP72 cell-surface expression was twice as high as that of LX-1 carcinoma cells that express HSP72 at the cell surface. A mild heat-shock (41.8 °C), however, led to a comparable elevation in the amount of HSP72 in both carcinoma cell lines. The rate of HSP72 induction after heat-shock in LX-1 cells was five times compared to 3 times in MX-1 carcinoma cells.

Furthermore, the thermoresistance of the two tumour cell lines was compared at 41.8 °C, 43 °C and 44 °C. The results derived from clonogenic cell survival assays are summarized in Fig. 4. For all different heating periods, ranging from 25 min to 150 min, the thermoresistance of LX-1 cells was always significantly higher than that of MX-1 carcinoma cells.

Discussion

Heat-shock proteins are essential for the survival of cells under normal as well as under stressful conditions like heat-shock, ethanol, arsenite, oxidative radical stress, steroid hormones etc. [9]. Members of the HSP70 family maintain mitochondrial proteins in an unfolded, translocationally competent state and prevent the denaturation of cytoplasmic proteins [2, 12]. Previous data from our group suggested that heat stress leads to a strong induction in the rate of synthesis of HSP72 [13], which results in a significant increase in the amount of cytoplasmic HSP72, the major heat-inducible form of the HSP70 group in sarcoma and in normal cells. Despite comparable levels of HSP72 in the cytoplasm, only sarcoma cells, and not normal cells, exhibit a HSP72 cell-surface expression. Therefore, we concluded that HSP72 cell-surface expression is not directly related to elevated cytoplasmic amounts of HSP72. This finding is supported by the data shown in the present study. Despite higher cytoplasmic amounts of HSP72 in MX-1 cells, HSP72 cell-surface expression was only found on LX-1 carcinoma cells. Even after non-lethal heat-shock, cell-surface expression could not be induced on MX-1 carcinoma cells (data not shown) thus indicating that cell-surface expression of HSP72 is independent of the amount of cytoplasmic HSP72. Jäättelä found that cytoplasmic overexpression of HSP70 rendered mouse fibrosarcoma cells more tumourigenic by allowing these cells to escape from tumour-necrosis-factor-mediated antitumour immune surveillance [6]. In our carcinoma cell system, cytoplasmic HSP72 overexpression does not correlate with the tumourigenicity in athymic mice since the MX-1 cells with high cytoplasmic HSP72 expression formed tumours less frequently and significantly more slowly than did LX-1 tumour cells that exhibited a low cytoplasmic HSP72 expression level (unpublished observation). Therefore, we conclude that the overexpression of cytoplasmic HSP72 in lung and mammary carcinoma cells does not affect tumourigenicity.

By comparing the sensitivity to lysis mediated by an IL-2-stimulated, adherent NK effector cell population it became obvious that LX-1, compared to MX-1 carcinoma cells, is significantly more sensitive to lysis. Since the MHC class I and class II expression patterns of both tumour cell lines were identical, we speculated that the HSP72 cell-surface expression is responsible for the different sensitivity to lysis. This hypothesis was confirmed by antibody-blocking studies using an HSP72-specific mAb: a strong inhibition of lysis was seen with LX-1 carcinoma cells that

express HSP72 on their cell surface but not with MX-1 carcinoma cells that lack HSP72 expression. Our results are in line with observations made by Tamura et al. [17], who postulated, for cytotoxic T cells, that the constitutively expressed HSP70 cognate protein acts as a tumour antigen. Furthermore, members of the HSP70 group have been shown to be involved in a number of pathological conditions, where they play a role as immunodominant antigens [3–5, 11, 15, 17, 19, 20].

In our previous studies we showed that heat- [14] or alkyl-lysophospholipid-induced [1] HSP72 cell-surface expression increases the immunogenicity of sarcoma and myelogenic lymphoma cells. Here we demonstrate that carcinoma cells that already express HSP72 molecules under physiological conditions on the cell surface, in comparison to carcinoma cells that lack HSP72 expression on the cell surface, are significantly more sensitive to lysis mediated by adherent NK cells. These data indicate that, apart from the immunogenic HSP72 determinant, no stress-inducible (i.e. heat or alkyl-lysophospholipids) factor is necessary for the increased sensitivity of tumour cells to lysis. However, it is still an open question whether cell-surface-expressed HSP72 molecules themselves act as immunogenic determinants [17] for adherent NK cells or whether tumour-specific peptides that are presented by HSP72 molecules [20] are also important for NK recognition.

Determination of the cell viability of both carcinoma cell lines after heat stress revealed that LX-1 carcinoma cells are more thermoresistant than MX-1 cells. Since the rate of induction and the total intracellular amount of HSP72 after heat stress were comparable in LX-1 and MX-1 cells, we speculate that the capacity to express HSP72 on the cell surface might be involved in this phenomenon. HSP72 that is localized in the plasma membrane of tumour cells might help to maintain membrane stability after thermal stress.

Despite this increased thermoresistance, HSP72 cell-surface expression renders tumour cells highly immunogenic against adherent NK cells that are stimulated with low-dose IL-2. Since normal cells lack the ability to express HSP72 on the cell surface, HSP72 might act as a tumour-specific recognition structure for a distinct NK effector cell population.

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References

1. Botzler C, Kolb HJ, Issels RD, Multhoff G (1996) Noncytotoxic alkyl-lysophospholipid treatment increases sensitivity of leukemic K562 cells to lysis by natural killer (NK) cells. *Int J Cancer* 65:633
2. Craig EA, Gambill BD, Nelson RJ (1993) Heat-shock proteins: molecular chaperones of protein biogenesis. *Microbiol Rev* 57:402
3. Ferrarini M, Heltai S, Zocchi MR, Rugarli C (1992) Unusual expression and localization of heat-shock proteins in human tumour cells. *Int J Cancer* 51:613

4. Fuller KJ, Issels RD, Slosman DO, Guillet J-G, Soussi T, Polla BS (1994) Cancer and the heat-shock response. *Eur J Cancer* 30A:1884
5. Heufelder AE, Wenzel BE, Bahn RS (1992) Cell surface localization of a 72 kilodalton heat-shock protein in retroocular fibroblasts from patients with Graves' ophthalmopathy. *J Clin Endocrinol Metab* 74:732
6. Jäättelä M (1995) Over-expression of hsp70 confers tumourigenicity to mouse fibrosarcoma cells. *Int J Cancer* 60:689
7. Hunt C, Morimoto RI (1985) Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proc Natl Acad Sci USA* 82:6455
8. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680
9. Lindquist S (1986) The heat-shock response. *Annu Rev Biochem* 55:1151
10. MacDonald HR, Engers HD, Cerottini JC, Brunner KT (1974) Generation of cytotoxic T lymphocytes in vitro. *J Exp Med* 140:718
11. Menoret A, Patry Y, Burg C, Le-Pendu J (1995) Co-segregation of tumour immunogenicity with expression of inducible but not constitutive hsp70 in rat colon carcinomas. *J Immunol* 155:740
12. Morimoto RI, Tissieres A, Georgopoulos C (1990) Stress proteins in biology and medicine. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Multhoff G, Botzler C, Wiesnet M, Müller E, Meier T, Wilmanns W, Issels RD (1995a) A stress-inducible 72-kDa heat-shock protein (HSP72) is expressed on the surface of human tumour cells, but not on normal cells. *Int J Cancer* 61:272
14. Multhoff G, Botzler C, Wiesnet M, Eissner G, Issels R (1995b) CD3⁺ large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72-kD heat-shock protein on human sarcoma cells. *Blood* 86:1374
15. Multhoff G, Hightower L (1996) Cell surface expression of heat shock proteins and the immune response. *Cell Stress Chaperones* 1 (3): 167
16. Stege GJ, Li GC, Li L, Kampinga HH, Konings AW (1994) On the role of hsp72 in heat-induced intranuclear protein aggregation. *Int J Hyperthermia* 10:659
17. Tamura Y, Tsuboi N, Sato N, Kikuchi K (1993) 70 kDa heat-shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumour immunity. *J Immunol* 151:5516
18. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76:4350
19. Tsuboi N, Ishikawa M, Tamura Y, Takayama S, Tobioka H, Matsuura A, Hirayoshi K, Nagata K, Sato N, Kikuchi K (1994) Monoclonal antibody specifically reacting against 73-kilodalton heat-shock cognate protein: possible expression on mammalian cell surface. *Hybridoma* 13:373
20. Udono H, Srivastava PK (1994) Comparison of tumour-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J Immunol* 152:5398
21. Vujanovic NL, Rabinovich H, Lee YJ, Herberman RB, Whiteside TL (1993) Distinct phenotypic and functional characteristics of human natural killer cells obtained by rapid interleukin 2-induced adherence to plastic. *Cell Immunol* 151:133
22. Weiss RA, Madaio MP, Tomaszewski JE, Kelly CJ (1994) T cells reactive to an inducible heat-shock protein induce disease in toxin-induced interstitial nephritis. *J Exp Med* 180:2239
23. Welch WJ, Feramisco JR (1984) Nuclear and nucleolar localization of the 72,000-dalton heat-shock protein in heat-shocked mammalian cells. *J Biol Chem* 259:4501

An 80-Kilodalton Antigen from *Histoplasma capsulatum* That Has Homology to Heat Shock Protein 70 Induces Cell-Mediated Immune Responses and Protection in Mice

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An extract of the cell wall and cell membrane from *Histoplasma capsulatum* yeast cells was assayed by Western blot (immunoblot) for reactivity with two monoclonal antibodies to heat shock protein 70. Four bands with molecular masses of 80, 66, 54, and 32 kDa bound both antibodies. The 80-kDa protein was isolated, analyzed for homology to heat shock protein 70, and tested for antigenicity and immunogenicity in C57BL/6 mice. The 80-kDa protein reacted with monoclonal antibody to heat shock protein 70. Sera from mice immunized with the antigen recognized *H. capsulatum* heat shock protein 70. Moreover, the amino-terminal sequence of the 80-kDa protein revealed substantial homology with heat shock protein 70 from several species. The 80-kDa protein induced delayed-type hypersensitivity responses in mice immunized with either viable yeast cells or antigen. Splenocytes from mice immunized with yeast cells or with antigen responded in vitro to the 80-kDa antigen. Immunization of mice with the antigen enhanced host resistance against a sublethal inoculum of *H. capsulatum* yeast cells, but it did not reduce the mortality of mice given a lethal challenge of yeast cells. Thus, this antigen manifests homology with members of the heat shock protein 70 family. Furthermore, the 80-kDa protein elicits cellular immune responses to *H. capsulatum*, and it mediates protective immunity.

Living organisms respond to adverse environmental conditions such as temperature elevation by a rapid increase in the synthesis of heat shock proteins (hsp). Production of these proteins presumably protects cells against the inimical effects of stress. Several families of hsp have been identified, and hsp 70 is one of the most abundant (19, 20).

Studies of hsp from the thermally dimorphic fungus *Histoplasma capsulatum* have focused on their role in the conversion of mycelia to yeast cells. This fungus exists as mycelia in soil but transforms into yeast cells once it has invaded mammalian tissues. The transition from mycelium to yeast cell is a heat-inducible event. When mycelia are exposed to elevated temperatures (34 to 40°C), there is intense synthesis of hsp, including hsp 70 (27). In addition, Caruso et al. (5) have shown that expression of the hsp 70 gene from *H. capsulatum* varies among strains that differ in pathogenicity and thermotolerance. Thus, hsp appear to play an important role in the mycelial phase-to-yeast phase conversion of *H. capsulatum*.

In recent years, it has been observed that members of the hsp family, including hsp 70 from several pathogenic microbes, are antigenic (for a review, see reference 33). Moreover, hsp of an invading microbe often are immunodominant targets of the cellular immune response as well as the humoral response (33). In the present study, we probed an extract of the cell wall and cell membrane of *H. capsulatum* yeast cells (CW/M) with monoclonal antibodies (MAb) to hsp 70 for the presence of immunoreactive bands. This extract was selected since previous work in this laboratory and others have demonstrated that it elicits in vivo and in vitro cellular immune responses and confers protective immunity (9, 11, 12).

Western blot (immunoblot) analysis of CW/M with MAb

to hsp 70 revealed the presence of four immunoreactive bands with molecular masses of 80, 66, 54, and 32 kDa. Since we previously reported that a murine CD4⁺ T-cell line recognized antigens in the extract that ranged in molecular mass from 69 to 82 kDa (12), the 80-kDa protein from CW/M that reacted with MAb to hsp 70 was isolated, analyzed for homology to members of the hsp 70 family, and tested for antigenicity and immunogenicity. The data demonstrate that this protein manifests homology to the hsp 70 family and induces cell-mediated immune responses in C57BL/6 mice. Furthermore, immunization with the 80-kDa antigen enhances resistance in mice given a sublethal challenge of *H. capsulatum* yeast cells.

MATERIALS AND METHODS

Mice. Male C57BL/6 mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Injection of mice with *H. capsulatum* yeast cells. Preparation of *H. capsulatum* yeast cells was performed as described previously (12). Mice were immunized with viable yeast cells by using the following regimen. On day 0, animals were injected subcutaneously (s.c.) with 10⁶ yeast cells. On day 14, mice received an intravenous (i.v.) injection of 6 × 10⁵ yeast cells, and on day 35 an intraperitoneal injection of 5 × 10⁶ yeast cells was administered. In experiments in which protective immunity was examined, mice were challenged i.v. with either a sublethal inoculum of 6 × 10⁵ yeast cells or a lethal inoculum of 4 × 10⁶ yeast cells.

Preparation of CW/M and the 80-kDa antigen. CW/M was isolated as described previously (12). Briefly, yeast cells were disrupted in a bead beater (Biospec Products, Bartlesville, Okla.), and the pellet was separated by differential centrifugation. The particulate material was boiled in an extraction buffer consisting of 125 mM Tris, pH 6.9, 6 M urea, 20 mM 2-mercaptoethanol, and 1% (vol/vol) Tween 20

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for 5 min and then incubated overnight at 4°C. The soluble material was separated by centrifugation and dialyzed against phosphate-buffered saline (PBS) for 36 h.

To isolate the 80-kDa antigen, 4 to 8 mg of CW/M was electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels that were 3 mm thick. Following electrophoresis, the gels were stained for 20 min at room temperature with 0.3% Coomassie brilliant blue that was dissolved in 10% glacial acetic acid and 30% isopropyl alcohol (16). The gels were destained with a solution containing 5% glacial acetic acid and 16.5% methanol for 2 to 3 h at 4°C (16). The 80-kDa band was excised and soaked in water for 2 h. The section of gel was minced and placed in an electroelutor (ELUTRAP; Schleicher & Schuell, Keene, N.H.). Elution was performed at room temperature for 8 h at 200 V in a buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. The purity of the eluted fraction was checked on SDS-polyacrylamide minigels, and if there were contaminating proteins, the material was electrophoresed and electroeluted a second time. SDS and Coomassie blue were extracted from the protein by the method of Konigsberg and Henderson (17). The antigen was suspended in PBS, pH 7.4, and filter sterilized prior to use. The protein content of the 80-kDa antigen was determined by the Bradford method (3).

ATP affinity chromatography and isolation of hsp 70 from *H. capsulatum*. Yeast cells were grown for 36 h at 37°C, heated for 2 h at 42°C, and then returned to 37°C for an additional 2 h. They were disrupted with a bead beater, and the cytosol was harvested by differential centrifugation. To isolate hsp 70, 20 to 25 mg of cytosol (3 to 5 ml) was applied directly to an ATP-agarose column (1.0 by 5.0 cm; Sigma Chemical Co., St. Louis, Mo.) (32). The column was washed with 40 ml of low-salt buffer (50 mM Tris, 0.1 NaCl, 0.1 M EDTA, 5 mM MgCl₂, 15 mM 2-mercaptoethanol [pH 7.5]) and then with 40 ml of high-salt buffer (low salt buffer containing 0.5 M NaCl). The column was rinsed with 10 ml of 10 mM ATP (Sigma Chemical Co.) in high-salt buffer. The ATP eluate was collected, dialyzed extensively against 2 mM ammonium bicarbonate, lyophilized, and resuspended in 0.5 ml of PBS, pH 7.2. Protein concentration was determined by the Bradford assay (3).

SDS-PAGE of the 80-kDa antigen. One microgram of antigen was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gradient polyacrylamide gel (19), and the gel was stained with silver (28).

Antibodies. Rat MAb 7.10, which recognizes members of the hsp 70 family of *Drosophila melanogaster* (18, 31), was kindly provided by Susan Lindquist, Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Ill. This immunoglobulin G (IgG) MAb chiefly reacts with denatured hsp 70 and does not bind hsp 70 in immunofluorescence or immunoprecipitation assays. Mouse MAb N27F3-4, specific for the human hsp 72/73, was provided by William Welch, University of California at San Francisco. This mouse IgG1 recognizes both the constitutive (72-kDa) and inducible (73-kDa) forms of human hsp 70 under both native and denaturing conditions. MAb N27F3-4 appears to recognize hsp72/73 isolated from all mammalian sources that have been examined (30). Control antibodies included a rat IgG and a mouse IgG1.

Western blot analysis of CW/M, the 80-kDa antigen, and hsp 70. One hundred twenty-five micrograms of CW/M or 1 µg of purified proteins was electrophoresed (10% polyacrylamide gels, 1 mm thick), transferred to nitrocellulose, and stained with Ponceau S. Lanes of nitrocellulose were cut and incubated in Tris-buffered saline (TBS), pH 7.5, containing

5% powdered milk for 1 h at room temperature to block nonspecific binding sites. Lanes were then incubated in 1 ml of TBS containing a 1:800 dilution of either MAb 7.10 or MAb N27F3-4 at 4°C overnight. In some experiments, the 80-kDa antigen or hsp 70 was incubated with a 1:1,000 dilution of sera from mice injected with the 80-kDa antigen or with bovine serum albumin (BSA). The strips were washed twice with TBS plus 2% Tween 20 for 10 min and incubated for 2 h with 1 ml of a 1:1,000 dilution of the appropriate peroxidase-labelled second antibody in TBS at room temperature. Blots were washed twice in TBS-detergent and developed with 0.6 mg of 4-chloro-1-naphthol (Bio-Rad Laboratories) per ml and hydrogen peroxide (0.015%).

Amino-terminal sequencing. Fifteen micrograms of the 80-kDa protein was electrophoresed in a 10% polyacrylamide minigel and electroblotted onto a polyvinylidene difluoride membrane (Schleicher & Schuell) in 3-[(cyclohexylamino)-1-propane-sulfonic acid] (CAPS) buffer, pH 11, at 250 mA for 1 h. The membrane was stained with Coomassie brilliant blue and destained with methanol (22). The band was excised and was sequenced with a gas phase microsequencer (Applied Biosystems; model 475A).

In vivo injection of mice with the 80-kDa antigen. The 80-kDa antigen was emulsified with an equal volume of complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) (Difco Laboratories, Detroit, Mich.). Mice were injected s.c. with 40 µg of antigen in CFA; 2 weeks later, they were injected s.c. with 40 µg of antigen in IFA.

Induction and measurement of DTH responses to the 80-kDa antigen. Groups of six mice immunized with viable *H. capsulatum* yeast cells or with the 80-kDa antigen were challenged intradermally with 1 µg of antigen in a volume of 0.05 ml. Footpad swelling was measured 24 h later. The delayed-type hypersensitivity (DTH) response was expressed as the mean footpad thickness in millimeters before and after antigen challenge and as a percent increase in footpad thickness (12). As a control, DTH was measured in age-matched littermates injected with an equal quantity of BSA in Freund's adjuvant. The percent increase was calculated as follows: [(thickness 24 h after injection of antigen - thickness before injection)/thickness before injection] × 100.

Organ culture for *H. capsulatum*. Spleens from mice infected with *H. capsulatum* were homogenized in PBS, and the homogenate was serially diluted. One hundred microliters of homogenate was dispensed onto plates containing brain heart infusion agar (2% [wt/vol] agar) supplemented with 5% (vol/vol) defibrinated sheep erythrocytes, 1% (wt/vol) glucose, and 0.01% (wt/vol) cysteine hydrochloride. Plates were incubated at 30°C, and CFU were enumerated after 7 to 10 days.

Splenocyte preparation and splenocyte proliferation assay. Spleen cells from mice were prepared as described previously (12). In the proliferation assay of splenocytes, 4 × 10⁵ cells in 0.2 ml of RPMI 1640, containing 10% fetal bovine serum and 10 µg of gentamicin per ml, and 0.05 ml of antigen (20 µg/ml) or medium were added to each well of a microtiter plate. For the proliferation assay of the murine T-cell line, 2 × 10⁴ resting cells in 0.1 ml of medium were incubated with 5 × 10⁵ irradiated splenocytes suspended in 0.1 ml of medium and 0.05 ml of antigen (20 µg/ml) or medium. Cultures of splenocytes were incubated for 144 h at 37°C in 5% CO₂, whereas cultures of the T-cell line were incubated for 72 h; 16 h before cell harvest, 0.5 µCi of [³H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) was added to each well. The final concentration

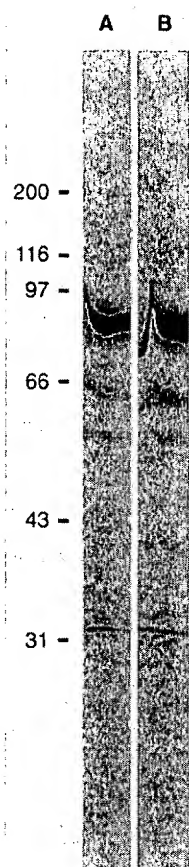


FIG. 1. Western blot analysis of CW/M with MAb to hsp 70. One hundred twenty-five micrograms of CW/M was electrophoresed in 10% gels, transferred to nitrocellulose, and reacted with a 1:800 dilution of either MAb 7.10 (lane A), which was raised against *Drosophila* hsp 70, or MAb N-27F3-4 (lane B), which was raised against human hsp 70. Molecular weight markers (10^3) are on the left.

of the 80-kDa protein in a well was 4 μ g/ml, which was an optimal concentration. Responses by cells to the 80-kDa antigen were considered positive when they were greater than or equal to three times the response of cells incubated in medium alone.

Statistics. The Mantel-Haenszel test was used to analyze survival of *H. capsulatum*-infected mice immunized with the 80-kDa antigen or BSA (24). The Wilcoxon rank sum test was used to analyze differences between groups. For both tests, $P < 0.05$ was considered significant.

RESULTS

Western blot analysis of CW/M. We sought to determine by immunoblot analysis if CW/M contained determinants recognized by MAb to members of the hsp 70 family. As shown in Fig. 1, MAb to *Drosophila* hsp 70 and to human hsp 70 reacted identically with CW/M. Four bands with various staining intensities were evident at molecular masses of 80, 66, 54 and 32 kDa. The curvilinear appearance of the 80- and 66-kDa bands was caused by intentional overloading of the gel with protein. This was done in order to ensure transfer of the many proteins in CW/M. Moreover, the patterns of

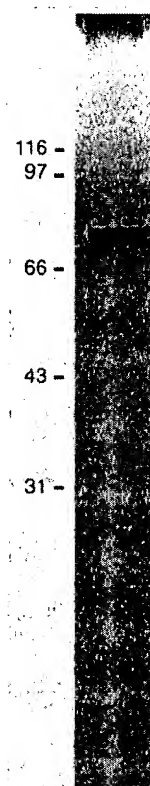


FIG. 2. SDS-PAGE analysis of the 80-kDa antigen. One microgram of protein was electrophoresed in a 10% gel and then stained with silver. Molecular weight markers (10^3) are on the left.

bands produced by incubating MAb to hsp 70 with CW/M were similar whether CW/M was produced from yeast cells subjected to heat shock (42°C for 2 h) or not (data not shown). No immunoreactive bands were apparent if CW/M was exposed to control antibodies. Thus, CW/M contained a determinant or determinants that were recognized by two disparate MAb to hsp 70.

Electrophoretic appearance of the 80-kDa protein and immunoblot analysis. We had reported previously that a murine T-cell line derived from C57BL/6 mice responded to a region of CW/M that encompassed 69 to 82 kDa (12). Therefore, it was postulated that the 80-kDa band that recognized MAb to hsp 70 might be one of the antigens within that region. The 80-kDa protein was isolated from SDS-PAGE gels by electroelution, and 1 μ g was electrophoresed. Figure 2 illustrates that the electroeluted protein migrated as a single band with a molecular mass of 80 kDa in a silver-stained gel. All assays of the 80-kDa protein were conducted with preparations that had a single band, as determined on silver-stained gels.

To determine whether the 80-kDa protein was recognized by MAb to hsp 70, 1 μ g of the protein was electrophoresed, transferred to nitrocellulose, and incubated with either rat MAb 7.10 or murine MAb N27F3-4 to hsp 70. The results of an immunoblot with MAb 7.10 are depicted in Fig. 3. The 80-kDa protein reacted with MAb to hsp 70. Thus, the 80-kDa protein possesses an epitope recognized by MAb to hsp 70.

Sera from mice immunized with the 80-kDa antigen recognize *H. capsulatum* hsp 70. Since the 80-kDa antigen con-

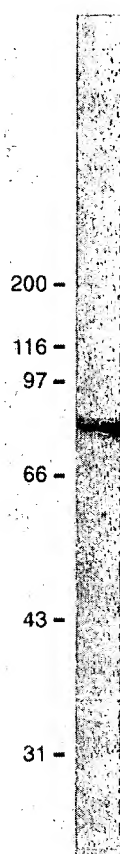


FIG. 3. Western blot of the 80-kDa antigen with MAb 7.10. One microgram of the 80-kDa antigen was electrophoresed in a 10% gel, transferred to nitrocellulose, and incubated with a 1:800 dilution of MAb 7.10. Molecular weight markers (10^3) are depicted on the left.

tained a determinant that was immunoreactive with MAb to hsp 70, the possibility was explored that sera from mice immunized with the 80-kDa antigen would recognize *H. capsulatum* hsp 70. Therefore, pooled sera from mice immunized with the 80-kDa antigen were tested for reactivity with *H. capsulatum* hsp 70.

Initially, we sought to isolate hsp 70 from *H. capsulatum*. Since hsp 70 binds to ATP (32), cytosol from yeast cells was subjected to ATP affinity chromatography. A 70-kDa protein eluted from the column with ATP (Fig. 4, left lane). This protein was transferred to nitrocellulose and immunoblotted with MAb 7.10. The 70-kDa protein reacted with this MAb (Fig. 4, right lane).

By Western blot analysis, sera from mice immunized with the 80-kDa antigen reacted with the 80-kDa antigen and hsp 70 (Fig. 5, lanes 1 and 3), whereas sera from mice immunized with BSA did not recognize either protein (Fig. 5, lanes 2 and 4). Thus, the 80-kDa antigen was serologically cross-reactive with hsp 70.

NH₂-terminal sequence of the 80-kDa antigen. To determine whether the 80-kDa antigen was homologous to members of the hsp 70 family at the amino acid level, the protein was subjected to microsequencing of the NH₂ terminus. Analysis of this sequence revealed a considerable homology at the amino acid level with hsp 70 from *Saccharomyces cerevisiae* (72%), *D. melanogaster* (72%), *Escherichia coli*

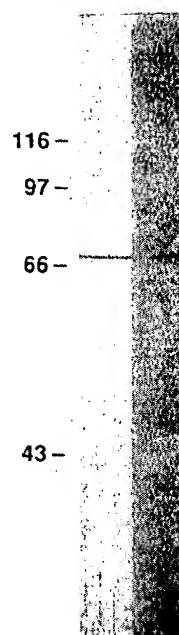


FIG. 4. Electrophoretic appearance of hsp 70 from *H. capsulatum* (left lane) and Western blot analysis of hsp 70 with MAb 7.10 (right lane). One microgram of hsp 70 was isolated by ATP affinity chromatography and electrophoresed in a 10% gel. The gel was stained with silver. One microgram was electrophoresed, transferred to nitrocellulose, and incubated with a 1:800 dilution of MAb 7.10. Molecular weight markers (10^3) are depicted on the left.

(56%), and humans (83%) (Table 1). Thus, the 80-kDa protein manifests homology to members of the hsp 70 family at the amino acid level and by serology.

The 80-kDa antigen induces DTH responses in mice immunized with viable yeast cells or with the 80-kDa antigen. Mice were injected with yeast cells or with 80 μ g of the 80-kDa antigen and tested for DTH to this antigen. The DTH response by either group of immunized mice was significantly greater ($P = 0.01$) than that by control animals (Table 2).

Splenocytes from mice injected with yeast cells or with the 80-kDa antigen proliferate in vitro in response to the 80-kDa antigen. Splenocytes from five individual C57BL/6 mice that had been immunized with viable *H. capsulatum* yeast cells or from five controls were tested for their capacity to recognize the 80-kDa antigen. The results for three mice are shown in Table 3. Spleen cells from all immunized mice proliferated in response to the antigen, whereas splenocytes from unimmunized animals did not.

Similarly, splenocytes from five individual C57BL/6 mice that had been immunized with 80 μ g of the 80-kDa antigen recognized this antigen. In contrast, spleen cells from all five mice given BSA did not respond to the 80-kDa antigen (Table 3).

The 80-kDa antigen but not hsp 70 from *H. capsulatum* causes proliferation of a T-cell line. Since the CD4⁺ T-cell line JC1 had responded to nitrocellulose-bound antigens encompassing 70 to 80 kDa (12), we determined whether the more purified 80-kDa antigen stimulated JC1. In several experiments, JC1 responded to the 80-kDa antigen. As an example, the mean (\pm standard error of the mean [SEM])

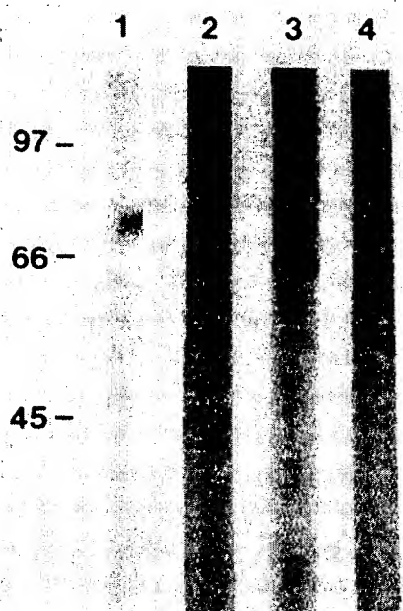


FIG. 5. Western blot analysis of the 80-kDa antigen and hsp 70, using sera from mice immunized with the 80-kDa antigen (lanes 1 and 3) or BSA (lanes 2 and 4). One microgram of the 80-kDa antigen or 1 μ g of hsp 70 was transferred to nitrocellulose and reacted with a 1:1,000 dilution of sera from mice. Lane 1, immune sera with 80-kDa antigen; lane 2, sera from BSA-injected mice with 80-kDa antigen; lane 3, immune sera with *H. capsulatum* hsp 70; lane 4, sera from BSA-injected mice with hsp 70. Molecular weight markers (10^3) are shown on the left.

proliferative response by JC1 to the 80-kDa antigen that 4 μ g/ml ($8,827 \pm 720$ cpm) exceeded that of cells cultured in medium alone ($1,070 \pm 73$ cpm).

In a separate set of experiments, we tested hsp 70 from *H. capsulatum* for its capacity to stimulate JC1. In three experiments, hsp 70 did not induce proliferation of JC1. In one experiment, the response by JC1 to hsp 70 at 5 μ g/ml ($1,487 \pm 200$ cpm) was not substantially higher than background ($1,203 \pm 197$ cpm). As a positive control, JC1 mounted a brisk response to 5 μ g of CW/M per ml ($20,621 \pm 3,734$ cpm).

Protective efficacy of the 80-kDa antigen. Groups of mice were immunized with 80 μ g of the 80-kDa antigen or with an equal amount of BSA. After immunization, the animals were challenged with a sublethal inoculum (6×10^5) of yeast cells. One week later, the animals were sacrificed, and the number

TABLE 1. Comparison of amino acid sequence of the 80-kDa antigen with sequences of hsp 70 from other species

Protein ^a	Amino acid sequence ^b
Hc 80-kDa Ag	A P A V - G I D L G T T Y S V V G I F
Sc hsp 70	<u>A</u> Y - G I D L G T T Y S C Y A H F
Dm hsp 70	<u>A</u> Y - G I D L G T T Y S C Y G V Y
Ec hsp 70	K I I G I D L G T T M S C Y A I M
Hu hsp 70	<u>A</u> A A Y - G I D L G T T Y S C Y G V F

^a Hc 80-kDa Ag, 80-kDa antigen from *H. capsulatum*; Sc hsp 70, hsp 70 from *S. cerevisiae*; Dm hsp 70, hsp 70 from *D. melanogaster*; Ec hsp 70, hsp 70 from *E. coli*; Hu hsp 70, human hsp 70.

^b Single-letter code used. Amino acids that are identical to those in the 80-kDa protein are underlined. Amino acid sequences were obtained from GenBank and reference 20.

TABLE 2. DTH responses to the 80-kDa antigen in immunized mice

Immunization ^a	Mean (\pm SEM) footpad thickness (mm) ^b	
	Before antigen challenge	After antigen challenge
None	2.00 \pm 0.03	2.06 \pm 0.01 (3) ^c
<i>H. capsulatum</i> yeast cells	2.20 \pm 0.06	3.15 \pm 0.11 ^d (43)
BSA, 80 μ g	2.05 \pm 0.04	2.09 \pm 0.04 (2)
80-kDa antigen, 80 μ g	2.05 \pm 0.01	2.40 \pm 0.03 ^d (17)

^a Mice were injected with viable yeast cells as described in Materials and Methods. One-half of the total dose of BSA or 80-kDa antigen was injected s.c. in CFA; 2 weeks later, the other half of the dose was given in IFA. Mice were tested 2 weeks later.

^b Mean \pm SEM for groups of six mice. Mice were tested with 1 μ g of the 80-kDa antigen.

^c Figures in parentheses indicate mean percent increase in footpad thickness from that measured immediately before antigen challenge.

^d Differences in footpad thickness between controls and immunized mice were significant at $P = 0.01$.

of *H. capsulatum* CFU in spleens was quantitated. The mean (\pm SEM) number of CFU in spleens of mice ($n = 10$) given the 80-kDa antigen ($43.0 \times 10^4 \pm 2.2 \times 10^4$ CFU) was significantly fewer ($P < 0.01$) than that in spleens of mice ($n = 10$) injected with BSA ($114.1 \times 10^4 \pm 6.4 \times 10^4$ CFU).

Subsequently, groups of six mice immunized with the 80-kDa antigen or with BSA were injected with a lethal inoculum (4×10^6) of yeast cells and were observed daily. In the 80-kDa-antigen-immunized group, three mice died on day 9, two mice died on day 10, and another mouse expired on day 11. In the controls, one mouse died on day 9, three died on day 10, and another died on day 11. Mantel-Haenszel analysis of survival revealed no statistical differences between groups ($P > 0.05$). Thus, the 80-kDa antigen reduced CFU in spleens of mice challenged with a lethal dose but failed to protect mice against a lethal challenge.

DISCUSSION

In this study, we examined the possibility that CW/M contained a determinant or determinants recognized by MAb to hsp 70. The rationale for this set of experiments was based on the previous finding that CW/M contained antigens whose molecular masses were similar to those of members of the hsp 70 family, that is, between 70 and 80 kDa (12). Western blot analysis revealed that two distinct MAb to hsp 70 produced identical patterns of reactivity with CW/M. Four bands with molecular masses of 80, 66, 54, and 32 kDa recognized both MAb. The profile of immunoblots suggests that these MAb recognized degradation products of hsp 70 in CW/M. This finding is not surprising since production of CW/M requires physical disruption of yeast cells. Disruption was performed in a buffer containing protease inhibitors and on ice. Nevertheless, it is still likely that some intact proteins are exposed to proteolytic enzymes and intense heat, which promote degradation.

Although there were several reactive bands, attention was focused on the 80-kDa protein because its molecular mass was in the region of that of nitrocellulose-bound antigen that caused stimulation of a murine T-cell line (12). Therefore, this protein was isolated from CW/M and tested for antigenicity and immunogenicity.

Serological studies strongly suggested that the 80-kDa protein shared a determinant or determinants with *H. cap-*

TABLE 3. Reactivity of splenocytes from immunized mice to the 80-kDa antigen in vitro

Immunization ^a	Stimulus ^b	[³ H]thymidine incorporation (mean cpm \pm SEM) ^c		
		Expt 1	Expt 2	Expt 3
None	Medium	4,007 \pm 131	5,502 \pm 106	94 \pm 4
	80-kDa antigen	4,675 \pm 306	4,607 \pm 329	134 \pm 28
<i>H. capsulatum</i> yeast cells	Medium	1,139 \pm 80	803 \pm 267	544 \pm 42
	80-kDa antigen	7,157 \pm 618	19,351 \pm 1,838	22,162 \pm 961
BSA, 80 μ g	Medium	538 \pm 47	782 \pm 126	186 \pm 21
	80-kDa antigen	495 \pm 156	896 \pm 158	346 \pm 70
80-kDa antigen	Medium	317 \pm 30	466 \pm 48	210 \pm 21
	80-kDa antigen	6,703 \pm 177	8,350 \pm 473	7,649 \pm 386

^a See Table 2, footnote a.^b The final concentration of the 80-kDa antigen was 4 μ g/ml.^c Mean \pm SEM of triplicate determinations.

sulatum hsp 70. In multiple experiments, we were unsuccessful in isolating the 80-kDa antigen from CW/M by ATP affinity chromatography, although *H. capsulatum* hsp 70 did bind to ATP (13). One potential problem was that CW/M is highly denatured; thus, the ATP-binding site for the 80-kDa protein may have been irrevocably altered. Therefore, to determine whether this antigen was homologous to hsp 70, amino-terminal sequencing was performed. A comparison of the amino acid sequences of the 80-kDa protein and hsp 70 from several species revealed a high degree of similarity. Thus, the 80-kDa antigen appears to be a homolog of hsp 70. The true identity of this antigen will be determined only once the gene has been cloned and sequenced.

The observation that a homolog of hsp 70, viz., the 80-kDa antigen, was present in the cell wall or cell membrane was unexpected since most proteins that are members of the hsp 70 family are present in the nucleus or cytoplasm of cells (23). However, there is precedent for expression of members of the hsp 70 family in the cell wall and cell membrane. A *Plasmodium falciparum* antigen that has 55% sequence homology to hsp 70 appears to reside on the surface of merozoites (1). A 75-kDa protein from *Chlamydia trachomatis* is present on the surface of elementary bodies (7). In addition, the cell membrane of cells of a murine B-cell lymphoma line, LK 35.2, contains a peptide-binding protein that is a member of the hsp 70 family (29). Preliminary experiments indicate by immunofluorescence that the 80-kDa antigen is not on the surface of *H. capsulatum* yeast cells (13). Studies are planned to identify more precisely the location of the 80-kDa protein in *H. capsulatum* yeast cells.

Additional studies of this antigen demonstrated that C57BL/6 mice immunized with viable yeast cells mounted a DTH response to the 80-kDa antigen and splenocytes from these mice proliferated in response to it. Furthermore, inoculation of the 80-kDa antigen in Freund's adjuvant induced cutaneous reactivity and in vitro blastogenic responses to the 80-kDa antigen. This antigen, therefore, is a target of the cellular immune response to *H. capsulatum* and induces cell-mediated immune responses. Moreover, since the antigen did not stimulate normal splenocytes, the data suggest that it is neither a mitogen nor a superantigen.

Members of the hsp 70 family are highly conserved at the amino acid level among eukaryotes and prokaryotes (20, 21, 23). The function of these proteins is to maintain polypeptides in an unfolded state in order to facilitate transport across membranes (6, 8, 10, 23). More recently, hsp 70 or homologs thereof from several species of parasites and bacteria have been shown to be antigenic. Thus, these

proteins from *P. falciparum* (2), *Schistosoma mansoni* (15), *Wucheria bancrofti* (26), and *Mycobacterium* sp. (4, 25) stimulate either a B-cell- or T-cell-mediated response or both. Since the 80-kDa antigen elicited both a cell-mediated and a humoral response, the data indicate that *H. capsulatum* also possesses an antigenic hsp 70 homolog.

Although the 80-kDa antigen manifests homology to hsp 70, it differs antigenically from the latter. The murine T-cell line responded to the 80-kDa protein but not to *H. capsulatum* hsp 70. Thus, the 80-kDa protein contains a peptide recognized by T cells that is not present in hsp 70. However, this finding does not exclude the possibility that cytosolic hsp 70 is antigenic since we did not assess whether splenocytes from mice immunized with *H. capsulatum* react with it.

In studies of protective immunity, the 80-kDa antigen significantly reduced the number of *H. capsulatum* CFU in spleens of mice given a sublethal injection of yeast cells i.v. However, it failed to prolong survival in mice given a lethal challenge of yeast cells. Why immunization with this antigen did not enhance survival among mice given a lethal dose of yeast cells is unclear. It is possible that larger quantities of the 80-kDa antigen might have significantly enhanced its protective capacity. These issues may not be resolved until there is a better understanding of host defense mechanisms that are operative during histoplasmosis. Nevertheless, it is important to recognize that this protein did enhance resistance to *H. capsulatum*.

This study extends the reported number of protective antigens in CW/M to two. There is the 80-kDa antigen and a previously isolated 62-kDa antigen (14). The latter, however, was able to confer protection in mice against a lethal challenge that was identical to the challenge employed in this study (14).

In summary, we have isolated an 80-kDa antigen from the cell wall and cell membrane of *H. capsulatum* yeast cells. This antigen is cross-reactive with MAb to hsp 70, and it manifests similarity at the amino acid level with hsp 70 from other species. This antigen induced cellular and humoral immune responses and conferred protective immunity. Thus, a homolog of hsp 70 from *H. capsulatum* is antigenic and immunogenic.

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REFERENCES

1. Ardeshtir, F., J. E. Flint, S. J. Richman, and R. T. Reese. 1987. A 75 kd merozoite surface protein of *Plasmodium falciparum* which is related to the 70 kd heat shock proteins. *EMBO J.* 6:493-499.
2. Bianco, A. E., J. M. Favolaro, T. R. Burkot, J. G. Culvenor, P. E. Crewther, G. V. Brown, R. F. Anders, R. L. Coppel, and D. J. Kemp. 1986. A repetitive antigen of *Plasmodium falciparum* that is homologous to heat shock protein 70 of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 83:8713-8717.
3. Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72:248-254.
4. Britton, W. J., L. Hellqvist, A. Basten, and A. S. Inglis. 1986. Immunoreactivity of a 70-kD protein purified from *Mycobacterium bovis* bacillus Calmette-Guerin by monoclonal antibody affinity chromatography. *J. Exp. Med.* 164:695-708.
5. Caruso, M., M. Sacco, G. Medoff, and B. Maresca. 1987. Heat shock protein 70 gene is differentially expressed in *Histoplasma capsulatum* strains with different levels of thermotolerance and pathogenicity. *Mol. Microbiol.* 1:151-158.
6. Chappell, T. G., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman. 1986. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell* 45:3-13.
7. Danilition, S. L., I. W. Maclean, R. Peeling, S. Winston, and R. C. Burnham. 1990. The 75-kilodalton protein of *Chlamydia trachomatis*: a member of the heat shock protein 70 family? *Infect. Immun.* 58:189-196.
8. Deshaies, R., B. Koch, M. Weiner-Washburne, E. Craig, and R. Scheckman. 1988. 70 kd stress protein homologues facilitate translocation of secretory and mitochondrial precursor polypeptides. *Nature (London)* 332:800-805.
9. Domer, J. E. 1976. In vivo and in vitro cellular responses to cytoplasmic and cell wall antigens of *Histoplasma capsulatum* in artificially immunized or infected guinea pigs. *Infect. Immun.* 13:790-799.
10. Finley, D., A. Crechanover, and A. Varshavsky. 1984. Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts 85. *Cell* 37:43-55.
11. Garcia, J. P., and D. H. Howard. 1971. Characterization of antigens from the yeast phase of *Histoplasma capsulatum*. *Infect. Immun.* 4:116-125.
12. Gómez, A. M., J. C. Rhodes, and G. S. Deepe, Jr. 1991. Antigenicity and immunogenicity of an extract from the cell wall and cell membrane of *Histoplasma capsulatum* yeast cells. *Infect. Immun.* 59:330-336.
13. Gomez, F. J., and G. S. Deepe, Jr. Unpublished observations.
14. Gomez, F. J., A. M. Gomez, and G. S. Deepe, Jr. 1991. Protective efficacy of a 62-kilodalton antigen, HIS-62, from the cell wall and cell membrane of *Histoplasma capsulatum* yeast cells. *Infect. Immun.* 59:4459-4464.
15. Hedstrom, R., J. Culpepper, R. A. Harrison, N. Agabian, and G. Newport. 1987. A major immunogen in *Schistosoma mansoni* infections is homologous to the heat shock protein HSP 70. *J. Exp. Med.* 165:1430-1435.
16. Hunkapillar, M. W., E. Lujan, F. Ostrander, and L. E. Hood. 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. *Methods Enzymol.* 91:227-236.
17. Konigsberg, W. H., and L. Henderson. 1983. Removal of sodium dodecyl sulfate by ion-pair extraction. *Methods Enzymol.* 91:254-259.
18. Kurtz, S., J. Rossi, L. Petko, and S. Lindquist. 1986. An ancient developmental induction: heat-shock proteins induced in sporulation and oogenesis. *Science* 231:1154-1157.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
20. Lindquist, S. 1986. The heat-shock response. *Annu. Rev. Biochem.* 55:1156-1191.
21. Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. *Annu. Rev. Genet.* 22:631-677.
22. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membrane. *J. Biol. Chem.* 262:10035-10038.
23. Pelham, H. R. B. 1986. Speculations on the functions of the major heat shock and glucose-related proteins. *Cell* 46:959-961.
24. Peto, R., M. C. Pike, P. Armitage, N. E. Breslow, D. R. Cox, S. V. Howard, N. Mantel, K. McPherson, J. Peto, and P. G. Smith. 1977. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. *Br. J. Cancer* 35:1-23.
25. Rees, A., A. Scoging, A. Mehler, D. B. Young, and J. Ivanyi. 1988. Specificity of proliferative response of human CD8 clones to mycobacterial antigens. *Eur. J. Immunol.* 18:1881-1887.
26. Selkirk, M. E., D. A. Denham, F. Partono, and R. M. Maizels. 1989. Heat shock cognate 70 is a prominent immunogen in Brugian filariasis. *J. Immunol.* 143:299-308.
27. Shearer, G., Jr., C. H. Birge, P. D. Yuckenberg, G. S. Kobayashi, and G. Medoff. 1987. Heat-shock proteins induced during the mycelial-yeast transition of strains of *Histoplasma capsulatum*. *J. Gen. Microbiol.* 133:3375-3382.
28. Tsang, V. C. W., K. Hancock, S. E. Maddison, A. L. Beatty, and D. M. Moss. 1984. Demonstration of species-specific and cross-reactive components of the adult microsomal antigens from *Schistosoma mansoni* (MAMA and JAMA). *J. Immunol.* 132:2607-2613.
29. Vanbuskirk, A., B. L. Crump, E. Margoliash, and S. K. Pierce. 1989. A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. *J. Exp. Med.* 170:1799-1807.
30. Vass, K. J., W. J. Welch, and T. S. Nowak, Jr. 1988. Localization of 70 kDa stress protein induction in gerbil brain after ischemia. *Acta Neuropathol.* 77:128-135.
31. Velazquez, J. M., and S. Lindquist. 1984. hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. *Cell* 36:655-662.
32. Welch, W. J., and J. R. Feramisco. 1985. Rapid purification of mammalian 70,000-dalton stress proteins: affinity of the proteins for nucleotides. *Mol. Cell. Biol.* 5:1229-1237.
33. Young, D. B., A. Mehler, and D. F. Smith. 1990. Stress proteins and infectious diseases, p. 131-165. In R. I. Morimoto, A. Tissières, and C. Georgopoulos (ed.), *Stress proteins in biology and medicine*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Heat Shock Protein 72 on Tumor Cells

A Recognition Structure for Natural Killer Cells¹

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Evidence is accumulating that members of the heat shock protein 70 (HSP70) family are found on the cell surface of certain tumor cells where they elicit a strong antitumor immune response. We demonstrated that HSP72, the major heat-inducible form of the HSP70 group, is located on the cell surface of ~60% of the human colon carcinoma cells CX2 with two different mAbs by indirect immunofluorescence, by electron microscopy, and by selective cell surface biotinylation. In an effort to analyze the role of HSP72 cell surface expression as a tumor-specific recognition structure within an "autologous" tumor system, the CX2 cells were separated into a stably HSP72 high expressing (CX+: >90%) and a stably HSP72 low expressing (CX-: <20%) subline. The expression "autologous" was written in parentheses to indicate that the colon carcinoma sublines CX+ and CX- derived from the original CX2 tumor cell line differ with respect to the cell surface expression pattern of HSP72, whereas they exhibit an identical cell surface expression pattern of MHC and cellular adhesion molecules (e.g., intercellular cellular adhesion molecule, neural cellular adhesion molecule, vascular cellular adhesion molecule). Within this "autologous" tumor cell system, we demonstrate that the sensitivity to lysis mediated by adherent non-MHC-restricted effector cells correlates ($p < 0.05$) with the amount of HSP72 that is expressed on the cell surface. Blocking studies using an HSP72-specific mAb revealed that HSP72 might act in an MHC-unrestricted manner as a tumor-specific recognition structure for a distinct NK cell population. *The Journal of Immunology*, 1997, 158: 4341–4350.

Molecular chaperones are required for a number of fundamental processes especially in the recovery from cellular stress. The best studied class of chaperones is the group of heat shock proteins (HSP)⁴ with a molecular mass of 70 kDa. Intracellularly they bind to and stabilize non-native conformations of other proteins and therefore inhibit aggregation of unfolded proteins or enable translocation across membranes (1, 2). It has been reported that HSP70 differentially localizes to the nucleus (3), to the cytosol, and to the cell surface of certain tumor cells (4, 5). Besides their intracellular chaperoning tasks, members of the HSP70 family appear to be highly immunostimulatory, e.g., in the pathology of several autoimmune diseases (6, 7), inflammatory processes (8, 9) associated with pathogens, and in the cellular

antitumor immune response in vivo and in vitro (10–16). However, the reasons why highly conserved proteins like heat shock proteins are immunodominant are still not clear. Presently four distinct mechanisms for the role of HSP in eliciting immune responses are discussed: 1) nonconserved HSP epitopes act as classical foreign Ags; 2) HSP cell surface expression is restricted to certain tissues or cell types; 3) molecular mimicry exists between HSP and non-HSP proteins; 4) HSP act as carrier molecules to present immunogenic peptides (17). For all immunostimulatory functions, accessibility of HSP to immunocompetent effector cells is required. Previous results from our laboratory (5, 21) suggest that HSP72, the major heat-inducible form of the HSP70 group, is expressed on the plasma membrane of human sarcoma cells after heat shock and that this heat-induced cell surface expression correlates to an increased sensitivity to lysis mediated by NK cells. The fact that HSP72 is expressed on the cell surface of only tumor cells, but not of normal cells, indicates that HSP72 might act as tumor-specific target structures for immunocompetent effector cells. Possible explanations for this tumor specificity could either be due to differences in the membrane composition and fluidity of membrane lipids (18) or due to a lower pH which is found in distinct tumor cell types. For diphtheria toxin, it has been demonstrated that low pH results in a conformational change of the globular protein into a membrane-bound protein (19).

In the present study, we demonstrate for human colon carcinoma cells that the heat-inducible HSP72 is expressed on the cell membrane on ~60% of the cells under physiologic conditions. A mild heat shock does not further enhance the percentage of cells that express HSP72 on the cell surface. Separation of CX2 cells into a stably HSP72 high expressing CX+ and a stably low expressing CX- cell line enabled us to study the role of HSP72 cell surface expression as an immunogenic determinant for NK-mediated lysis within an "autologous" tumor cell system.

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⁴ Abbreviations used in this paper: HSP, heat shock protein; ICAM, intercellular cellular adhesion molecule; VCAM, vascular cellular adhesion molecule; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum.

Table I. Antibodies used for phenotypic characterization of effector and tumor cells, antibody blocking studies, and immunoprecipitation (methods described below), and cell sorting

Antibody	Specificity	Isotype	Source
Isotype control		IgG2a	Dianova, Hamburg, Germany
Isotype control		IgG1	Dianova ^a
W6/32	MHC class I	IgG2a	J. Johnson, LMU, Munich, Germany ^b
L243	HLA-DR	IgG2a	J. Johnson
Anti-CD54	ICAM	IgG1	Dianova
Anti-CD56	NCAM	IgG1	Dianova
Anti-CD16	Fcγ RIII	IgG1	Dianova
Anti-CD57	HNK1	IgM	Dianova
VCAM-1	VCAM	IgG1	Dianova
Anti-CD3	T cells	IgG1	Dianova
OKT3	T cells	IgG2a	ATCC ^c
Anti-CD14	Monocytes	IgG2a	Dianova ^c
RPN1197	HSP72	IgG1	Amersham, Braunschweig, Germany ^{a,c}
3A3	HSP72	IgG1	S. Fox, Northwestern University, Chicago, IL, USA ^{a,c}
CD3/CD16 + 56	T/NK cells	IgG1/IgG1	Becton Dickinson, Heidelberg, Germany
CD45/CD14	Lymphocytes	IgG1/IgG2b	Becton Dickinson
FITC rabbit anti-mouse		Ig	Dako

^a Antibody blocking studies.^b Immunoprecipitation.^c Cell sorting.

Materials and Methods

Cell culture

The human colon carcinoma cell line CX2 (Tumorbank DKFZ, Heidelberg, Germany) and the subclones CX+ and CX- were cultured in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Life Technologies) and antibiotics.

Heat treatment

Exponentially growing CX2 cells were treated with a nonlethal temperature (41.8°C) for 2 h in a temperature-controlled water bath (Haake E3, Karlsruhe, Germany) and were then incubated at 37°C for 12 h. The cell viability of the heat-treated cell was always >95% as determined by trypan blue exclusion and propidium iodide staining.

Generation of adherent NK effector cells

As previously described (20, 21), PBMC derived from four donors were separated into a nonadherent CD3⁺ T cell fraction and an adherent CD3⁺ (CD16/CD56 positive)-NK cell fraction 1) by sequential incubation on plastic tissue culture flasks containing medium supplemented with rIL-2 (100 IU/ml) and 2) by magnetic bead separation and cell sorting as described below using mAb directed against CD3 and CD14 (22). The CD3⁺ NK cell fraction which was used for further investigations on d4 after stimulation with rIL-2 (100 IU) was phenotypically characterized by flow cytometric analysis using anti-CD3/CD16/CD56 and CD14/CD45 double-stained Abs as described below. The NK cell fraction was devoid of B cells, monocytes, and T cells as determined by flow cytometry.

mAb, indirect immunofluorescence, FACScan analysis, cell sorting

The Abs described in Table I were used for phenotypic characterization of effector and tumor cells (FACScan instrument, Becton Dickinson, Heidelberg, Germany), for cell sorting (FACStar^{Plus} Instrument, Becton Dickinson), for Ab blocking studies, and for immunoprecipitation.

The flow cytometric studies and the cell sorting experiments of the tumor cells were performed with exponentially growing cells and at comparable cell densities. Viable cells were incubated with the respective Abs shown above at a final concentration of 1 to 5 μg/0.5 to 1 × 10⁶ cells for 30 min at 4°C. All Abs used for FACScan analysis and cell sorting contained 0.1% sodium azide; the Abs used for blocking studies were sodium azide free. After incubation with the primary Abs, the cells were washed twice with PBS/10% FCS solution and incubated with a second FITC-conjugated rabbit anti-mouse Ig Ab (Dako, Hamburg, Germany) for another 30 min at 4°C.

The percentage of positively stained cells was defined as the difference of the number of specifically stained, viable cells (propidium iodide negative) minus the number of cells stained with an isotype-matched control

Ab on a FACScan instrument. The data obtained from FACScan analysis represent the mean values of at least four independent experiments.

For cell sorting, 5 × 10⁶ tumor cells were stained with the HSP72-specific mAbs (RPN1197, 3A3) and rabbit anti-mouse FITC according to the protocol described for flow cytometry. Then, on a FACStar^{Plus} instrument, tumor cells that expressed HSP72 on the cell surface were separated from cells that did not express HSP72 on the cell surface.

The specificity of the HSP72 mAb RPN1197 (Amersham) that was used in the present study has been demonstrated by Western blot analysis following two-dimensional and one-dimensional SDS-PAGE in a previous study (5): the Ab selectively detects HSP72, the major heat-inducible form of the HSP70 group, and does not cross-react with the constitutive form, HSP73. Cytoplasmic HSP72 expression was detectable with the Abs RPN1197 (Amersham, Braunschweig, Germany), 3A3 (S. Fox, Northwestern University, Chicago, IL), C92F3A-5 (StressGen Biotechnologies Corp., Victoria, Canada), and 5G10 (PharMingen). Cell surface staining, cell sorting, and surface biotinylation experiments with tumor cells were performed with mAb RPN1197 and mAb 3A3. The HSP72 high expressing (CX+) and the low expressing (CX-) colon carcinoma sublines were cultured separately after sorting.

Cell synchronization

Cell synchronization at the G₁-S boundary of the cell cycle was achieved with an aphidicolin (Sigma Chemical Co., St. Louis, MO) block. One day after cells were plated, the medium was removed and replaced with medium containing aphidicolin at a final concentration of 5 μg/ml. After a 24-h incubation, the cells were washed three times with PBS and refed with fresh medium (RPMI 1640 supplemented with 10% FCS).

Cell cycle analysis

For the simultaneous determination of HSP72 cell surface expression and DNA content, cells were stained with HSP72 mAb and FITC before fixation and permeabilization. DNA content of CX2 cells was measured by flow cytometry according to a method described elsewhere (21, 23).

Electron microscopy

PBS-washed CX2, CX-, and CX+ tumor cells either untreated or after heat shock (42°C for 2 h, 37°C for 12 h) were fixed in 8% paraformaldehyde in HEPES (250 mM) buffer for 1 h. After two washes, free aldehyde groups were quenched with 50 mM NH₄Cl for 10 min. The cell pellets were kept suspended in a volume of 2.1 M sucrose in polyvinylpyrrolidone (17%) at 20°C for 30 min for cryoprotection. Then the cell pellets were frozen in liquid nitrogen, and ultrathin sections (70 nm) were cut at -100°C on an Ultracut E microtome (Reichert-Jung FC4E) using a glass knife and mounted on 150 mesh Parlodion (Mallinckrodt Specialty Chemicals, Chesterfield, MO)-coated nickel grids. Immunogold labeling of HSP72 was performed with ultrasmall gold probes followed by silver enhancement according to the method described by Danscher (24). Briefly,

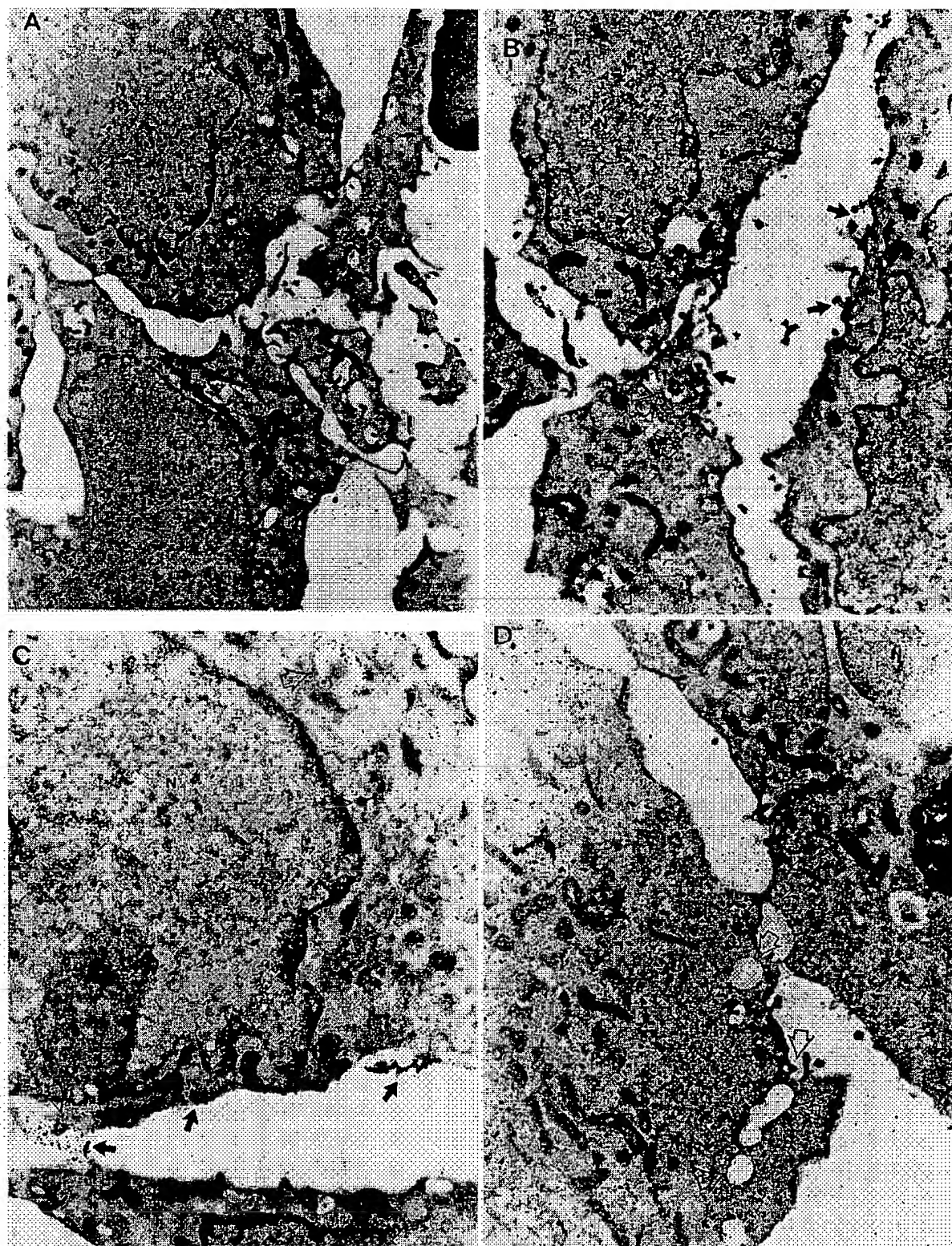


FIGURE 1. Cryo-ultramicrotomy image of labeled HSP72 on CX2 human colon carcinoma cells. The frozen sections were exposed to anti-HSP72 mAb (RPN1197, Amersham) followed by ultrasmall gold that was enhanced with silver. A represents control gold labeling and silver enhancement without primary Ab. As demonstrated in B and C, the labeling of HSP72 is not restricted to subcellular compartments in the cytoplasm and nucleus; HSP72 can be detected also in vesicle-like structures and on cellular projections of the plasma membrane. In C, two neighboring cells are shown that differ with respect to the cell surface expression of HSP72: the cell in the upper part exhibits HSP72 in the cytoplasm and on the cell surface; the cell in the lower part does not express HSP72 on the cell surface. An additional CX2 tumor cell that does not express HSP72 on the cell surface is shown in D. Filled arrows denote enrichment of HSP72 on cellular projections on the plasma membrane; open arrows mark vesicle-like structures; N indicates the nucleus. A and B, $\times 16,000$; C and D, $\times 35,000$.

the grids were rinsed and blocked in 0.1% acetylated BSA buffer. Then the grids were incubated with HSP72 mAb (RPN1197, dilution 1:200) overnight at 4°C. After the cells were washed, they were incubated in AURION goat anti-mouse IgG/IgM (Amersham) GP-Ultra small gold in 0.1% BSA buffer diluted 1:75 for 3 h at room temperature. Nonspecific binding was blocked by extensive washing in 0.1% acetylated BSA buffer. An additional fixation in 2.0% glutaraldehyde in PBS was performed after immunostaining. After silver enhancement, the sections were stained in uranyl acetate/methyl cellulose and viewed in a Zeiss EM 10CR electron microscope. This method is an approach that applies to the localization of Ags that are localized in both intracellular and extracellular compartments.

Selective cell surface biotinylation and immunoprecipitation

For detection of HSP72 cell surface expression, viable cells (2×10^7) were biotinylated with biotin ester (RPN2202, Amersham) for 30 min at 4°C. After the cells were washed, they were lysed for 20 min in ice-cold lysis buffer (0.1% Nonidet P-40; 250 mM NaCl; 25 mM Tris-HCl, pH 7.5; 5 mM EDTA; 2 µg/ml aprotinin; 100 µg/ml PMSE). Extracts were clarified by microcentrifugation for 15 min at 4°C and immunoprecipitated with HSP72 mAb (RPN1197, 10 µl, or 3A3, 5 µg) or with W6/32 mAb (2 µg) and 50 µl of gamma-bind plus protein G-Sepharose beads for 3 h at 4°C. Then the conjugated Sepharose beads loaded with Ag were washed three times in ice-cold lysis buffer and resuspended in 75 µl of sample buffer (200 mM DTE, 20% glycerol, 0.12 M Tris, bromophenol blue). Proteins from the Sepharose beads were separated on a 10% SDS-PAGE, blotted on a polyvinylidene difluoride (PVDF) membrane, and detected by enhanced chemiluminescence (ECL) after incubation with a secondary peroxidase-conjugated streptavidin Ab.

Cytoplasmic HSP72 was detected by reprobing of the membranes with mAb directed against HSP72, as described below.

SDS-PAGE and Western blot analysis

Equal protein amounts (10 µg) of cell lysates and 50 ng of rHSP72 (Stress-Gen Biotechnologies Corp.) were electrophoresed on 10% SDS-PAGE as described previously (5) according to the method of Laemmli (25). After SDS-PAGE, the proteins were transferred to Immobilon PVDF membranes (Millipore Corp., Bedford, MA) following a standard protocol (26). Nonspecific binding of the membrane was blocked with 5% skim milk in PBS. The blots were incubated with the HSP72 primary Ab (Amersham) and the secondary Ab (goat anti-mouse IgG peroxidase conjugated, Bio-Rad, Germany) each for 1 h. Immune complexes were detected using the ECL detection system (Amersham, Braunschweig, Germany).

For biotinylated proteins, 20 µl of the samples were electrophoresed and transferred to Immobilon PVDF membranes (Millipore Corp.). The membranes were blocked for 1 h at room temperature in PBS containing 5% blocking reagent (Amersham, NIF833) and 1% Tween-20 (Sigma Chemical Co.), washed three times for 10 min in PBS containing 1% Tween-20, and probed for 1 h with horseradish peroxidase-conjugated streptavidin (Amersham) diluted 1:1500 in PBS containing 1% Tween-20. The biotinylated proteins were detected by the ECL system.

As a control, rHSP72 protein (50 ng) either untreated or after partial trypsin digestion (5 min) was run on SDS-PAGE and detected by the ECL system.

Cytotoxicity assay

The specificity of rIL-2-activated, purified NK effector cells (21) was monitored in a standard ^{51}Cr release assay (27). The CX2, CX+, and CX- tumor cell lines were used as target cells. Briefly, the tumor cells were labeled with a stock solution of 5 mCi/ml ^{51}Cr for 2 h at 37°C. After three washing steps in RPMI 1640/15% FCS, the cells were counted and adjusted to 1×10^4 cells/ml. Then the target cells were coincubated with the effector cells at varying E:T ratios ranging from 40:1 to 5:1 for 4 h at 37°C. The percentage of specific lysis was calculated as: [(experimental release - spontaneous release)/(maximum release - spontaneous release)] \times 100. The percentage of spontaneous release was calculated as: (spontaneous release/maximum release) \times 100 and was always <20% for each target cell.

Ab blocking study

The inhibition assays were performed by preincubation of the target cells with the following Abs at a final concentration of $5 \mu\text{g}/1 \times 10^6$ cells for each Ab: RPN1197 (anti-HSP72); an isotype-matched IgG1 control Ab; and an MHC class I (W6/32) Ab (28). After incubation, the cells were washed twice with RPMI 1640/10% FCS, and the cytotoxicity assay was performed as described above.

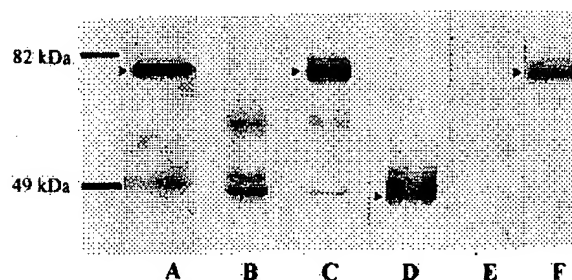


FIGURE 2. Western blot analysis of HSP72 expressed on the plasma membrane of biotinylated CX2 tumor cells. Lane A, rHSP72 protein (50 ng) detected by the specific HSP72 mAb; lane B, rHSP72 protein after trypsin digestion; lanes C and D, immunoprecipitates obtained with anti-HSP72 (lane C) or anti-MHC class I mAb (lane D) from 0.1% Nonidet P-40 lysates after selective cell surface biotinylation; lane E, control immunoprecipitates after selective cell surface biotinylation of MX-1 tumor cells that do not express HSP72 on the cell surface (21); lane F, after washing, the membrane shown in lane E was reprobed with anti-HSP72 mAb to identify cytoplasmic HSP72 proteins. Left margin, Molecular mass standard: 82 and 49 kDa. Arrowheads mark proteins with 72- and 45-kDa bands, respectively.

Statistical analysis

The significance of differences between different experimental values was assessed by means of Student's *t* test.

Results

Cell surface expression and subcellular distribution of HSP72 was studied by electron microscopy using ultrasmall gold particles and silver enhancement as described in *Materials and Methods*. The background staining of CX2 colon carcinoma cells without incubation with the first Ab is shown in Figure 1A. The immunoelectron micrograph presented in Figure 1B shows that HSP72 (indicated as black dots) is associated with the plasma membrane of CX2 colon carcinoma cells. Since the membrane was permeable before Ab incubation, HSP72 is also detectable in the cytoplasm and in the nucleus. The results of HSP72 distribution in CX2 colon carcinoma cells that differ with respect to the cell surface expression is shown in Figure 1C: the cell in the upper part of the micrograph exhibits HSP72 molecules in the cytoplasm, in vesicle-like structures, in the nucleus, and on cellular projections of the plasma membrane; the cell in the lower part does not express HSP72 on the plasma membrane and in vesicles. Figure 1D shows additional CX2 tumor cells that do not express HSP72 on the cell surface; HSP72 is found in the cytoplasm but not in vesicle-like structures.

Furthermore, HSP72 cell surface expression on CX2 tumor cells was shown by selective cell surface biotinylation. A comparison of recombinant HSP72 (rHSP72; Fig. 2, lane A) with biotinylated plasma membranes of CX2 cells reveals that intact HSP72 molecules can be immunoprecipitated from the cell surface of CX2 cells (Fig. 2, lane C); lane B depicts partial digestion products of rHSP72 protein after trypsin digestion (stained with HSP72 mAb 3A3) that correspond to the additional protein bands found in the biotinylated sample (lane C). Figure 2, lane D, represents MHC class I molecules immunoprecipitated with the mAb W6/32. As a negative control, the biotinylated immunoprecipitation products of tumor cells that do not express HSP72 on the cell surface (MX-1, 21) are shown in lane E. Reprobing of this membrane with HSP72 mAb (3A3) exhibits a 72-kDa protein band representing cytoplasmic HSP72.

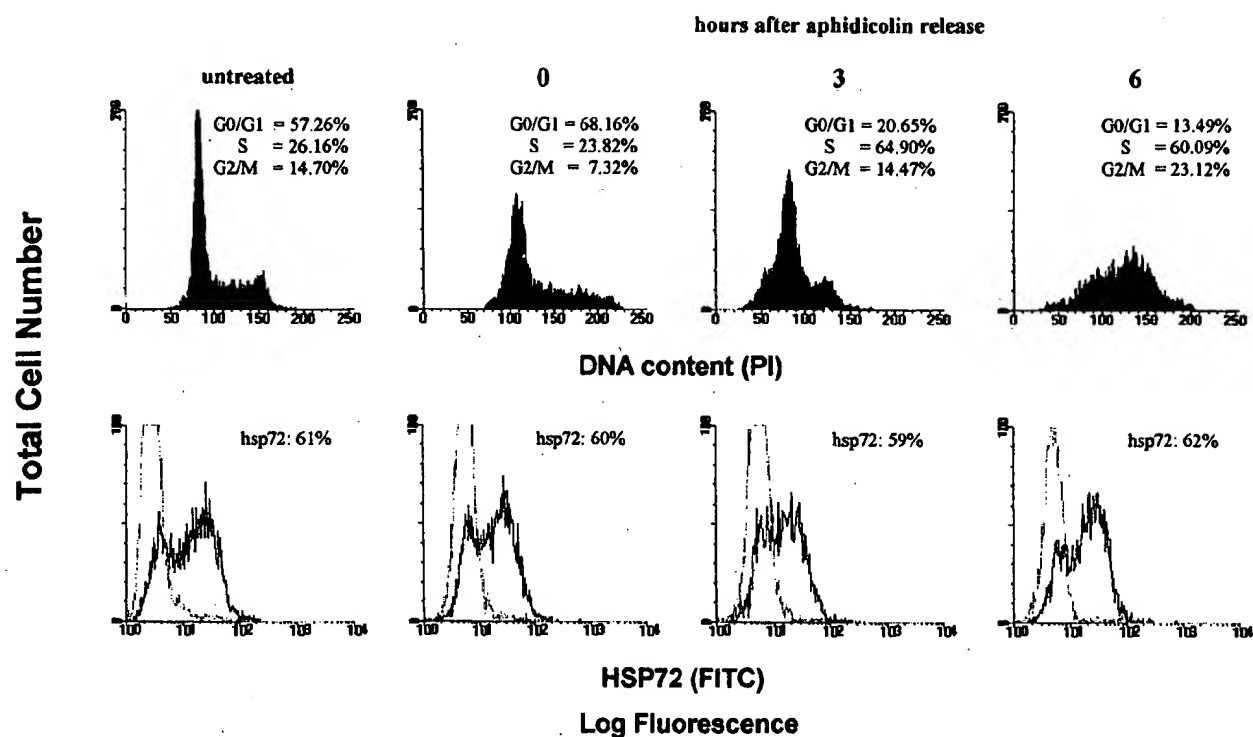


FIGURE 3. Simultaneous determination of the cell cycle distribution and HSP72 cell surface expression in untreated and synchronized CX2 colon carcinoma cells 0, 3, and 6 h after aphidicolin release. *Upper row*, Flow cytometry histogram profiles showing the DNA content based on propidium iodide fluorescence. *Lower row*, Flow cytometry histogram profiles showing the level of HSP72 cell surface expression during different phases of the cell cycle; dotted line, isotype-matched negative control Ab; solid line, HSP72 mAb.

Table II. Comparative flow cytometric analysis of MHC, adhesion molecules, and HSP72 cell surface expression (RPN1197; 3A3) on CX2, CX+, and CX- carcinoma cells (n = 5)

	MHC I	MHC II	ICAM-1	VCAM	HNK1	NCAM	HSP72
Antibody	W6/32	L243	Anti-CD54	Anti-CD106	Anti-CD57	Anti-CD56	Anti-HSP72
CX2	+++ ^a	—	++	—	—	+	++
	(99 ± 0.5)	(5 ± 3.7)	(58 ± 2.3)	(8 ± 0.5)	(7 ± 1.5)	(21 ± 4.6)	(66 ± 6.8)
CX+	+++	—	++	—	—	+	+++
	(99 ± 0.8)	(10 ± 0.5)	(68 ± 7.7)	(11 ± 3.5)	(12 ± 6.6)	(26 ± 2.3)	(93 ± 3.4)
CX-	+++	—	+	—	—	+	—
	(99 ± 0.5)	(10 ± 4.7)	(48 ± 4.5)	(12 ± 4.5)	(10 ± 4.0)	(24 ± 8.8)	(18 ± 6.0)

^a +++, >90%; ++, >50%; +, >20%; —, <20%.

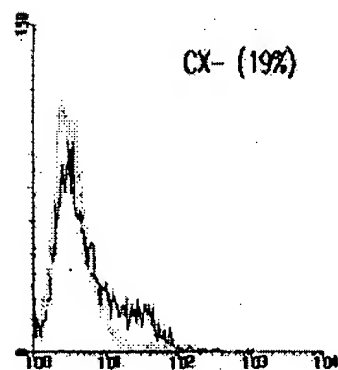
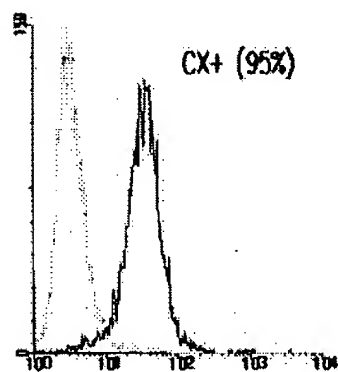
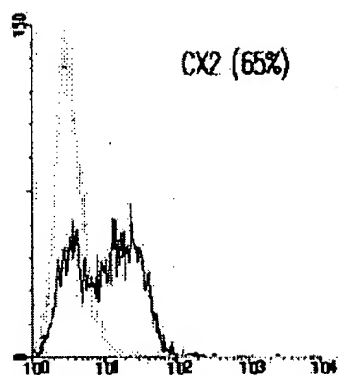
Cell surface expression of HSP72 on CX2 cells was quantified by indirect immunofluorescence studies using the HSP72-specific mAb RPN1197 and 3A3 followed by FACScan analysis. Under physiologic conditions, HSP72 molecules were constantly expressed on ~60% of the cells as shown in Figure 3. In an attempt to answer the question as to whether HSP72 cell surface expression is cell cycle associated, flow cytometry was used to measure DNA content and heat shock protein cell surface expression simultaneously using aphidicolin-synchronized CX2 cells. Despite clear differences in the cell cycle distribution 0, 3, and 6 h after the release of aphidicolin, the HSP72 cell surface expression remained uniform (Fig. 3). For further analysis of this phenomenon, the CX2 carcinoma cell line was separated into a HSP72 high expressing (CX+: >90%) and a HSP72 low-expressing (CX-: <20%) subline. Representative histograms of the phenotypic characterization of HSP72 cell surface expression on CX2, CX+, and CX- cell lines are shown in Figure 4. Repeated cell sorting experiments revealed that the CX- fraction always contains low concentrations of CX+ cells; however, directly after sorting no CX+ cells were

detectable, thus indicating that capping phenomena are responsible for this finding. By comparative flow cytometric analysis of MHC, adhesion molecules, and HSP expression, we demonstrate that the original cell line CX2, the CX+ and the CX- sublines differ only in their capacity to express HSP72 on their cell surface but not in any of the other tested cell surface markers (Table II). At comparable cell densities, CX2, CX+, and CX- cells revealed comparable ICAM-1 expression levels. This cell surface expression pattern remained stable for at least 30 cell passages.

In contrast to the profound differences in the cell surface expression of HSP72 demonstrated by flow cytometry and electron microscopy, the cytoplasmic amount of HSP72 in the original CX2 cell line and the sublines CX+ and CX- were comparable under physiologic conditions and after a mild heat stress (41.8°C, 2 h; 37°C, 12 h). As shown in Figure 5, a mild heat shock results in a comparable induction rate of HSP72 (about 13-fold) in all three cell lines.

To study the subcellular distribution of HSP72 comparative electron microscopic analysis of sorted CX+ and CX- cells either

Total Cell Number



HSP72 (FITC) Log Fluorescence

FIGURE 4. HSP72 cell surface expression on CX2 colon carcinoma cells after flow cytometric cell sorting using HSP72-specific mAb. Results are expressed as log green fluorescence intensity vs relative cell numbers. Dotted lines represent the isotype-matched negative control; solid line, HSP72 mAb.

untreated (Fig. 6, A and C) or after heat shock (Fig. 6, B and D) were performed. The total cytoplasmic amount of HSP72 was increased about equally in both tumor sublines; however, the sub-cellular distribution differs significantly: an enrichment of HSP72

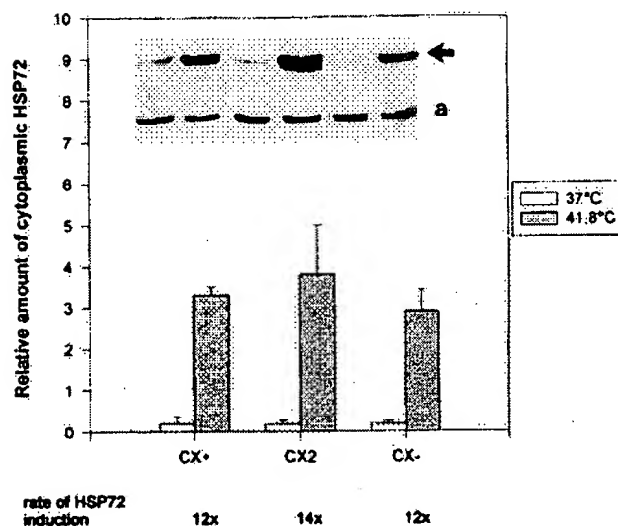


FIGURE 5. A, HSP72 immunoblot of cytoplasmic lysates of CX+, CX2, and CX- carcinoma cells under physiologic conditions (open bars) and after a mild heat stress at 41.8°C for 2 h, followed by a recovery period at 37°C for 12 h (hatched bars). HSP72 protein amounts (arrow) were related to actin (a). Equal protein amounts (10 μ g) of the cell lysates were run on 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose. The 72-kDa protein band was demonstrated with the HSP72-specific mAb and detected with the ECL system. The immunoblots were quantified by laser densitometry. The results represent the mean of at least three independent experiments. Inset, One representative immunoblot. The relative rate of HSP72 induction is indicated below each panel.

in vesicle-like structures was predominantly found in CX+ cells under physiologic conditions and after heat stress (Fig. 6, A and B); CX- cells do not accumulate HSP72 in vesicle-like cellular compartments under both conditions (Fig. 6, C and D). The HSP72 cell surface-staining patterns of untreated and heat-treated CX+ cells were comparable; no cell surface expression of HSP72 is detectable on CX- cells even after heat shock.

The role of the differential cell surface expression of HSP72 within an "autologous" colon carcinoma cell system was analyzed with respect to the sensitivity to lysis mediated by adherent NK cells. As demonstrated in Figure 7A and Table III, the sensitivity to lysis against NK cells was significantly increased (1.794 ± 0.425 ; $p = 0.033$) in the HSP72 high expressing CX+ subline compared with the low expressing CX- subline. A comparison of the sensitivity to lysis of CX- cells to the original CX2 tumor cell line revealed an increase in lysis of 1.279 ± 0.178 ($p = 0.052$). Furthermore, heat shock results neither in an increased cell surface expression of HSP72 (Figs. 5 and 6) nor in an elevated sensitivity to lysis mediated by NK cells (data not shown).

By Ab-blocking assays, the sensitivity to lysis mediated by NK cells could be positively correlated to the amount of HSP72 that is expressed at the cell surface (Fig. 7B). Using the HSP72-specific mAb, the strongest inhibition of lysis was found with CX+ cells, an intermediate inhibition of lysis was seen with the original CX2 tumor cell line, and no inhibition of lysis was detectable with the CX- cell subline. An IgG1 isotype-matched control Ab and an MHC class I-specific Ab (data not shown) had no inhibitory effect on the lysis pattern of CX2, CX+, and CX-. A calculation of the correlation of HSP72 cell surface expression and the sensitivity to lysis mediated by NK cells is demonstrated by statistical analysis (Table IV). A significant correlation of HSP72 expression and cell lysis was found for CX+ ($p = 0.001$), CX2 ($p = 0.005$), and

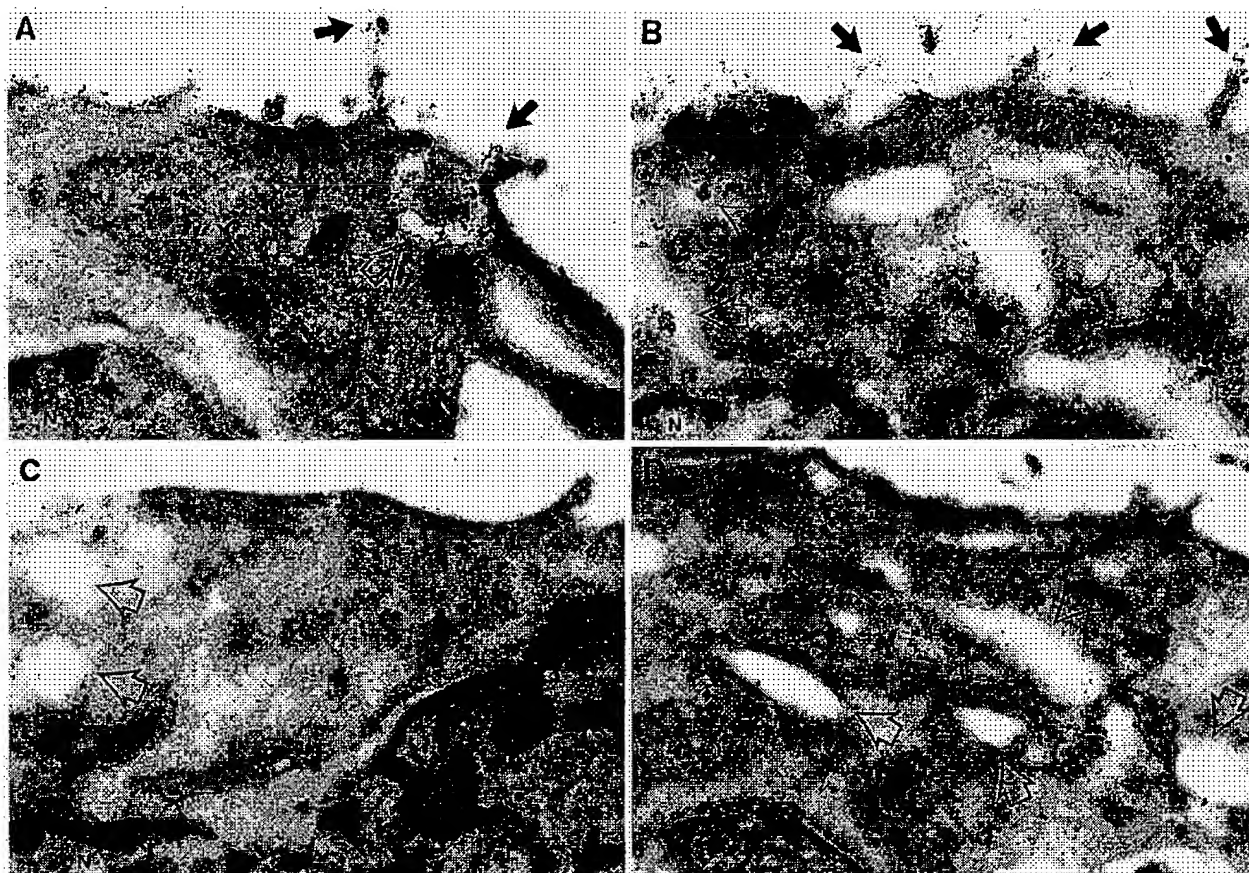


FIGURE 6. Comparative cryo-ultramicrotomy image of labeled HSP72 on CX+ and CX- colon carcinoma sublines either untreated or after heat shock (42°C for 2 h; 37°C for 12 h). The micrographs illustrate the subcellular distribution of HSP72 in CX+ and CX- cells under physiologic conditions (A and C) and after heat shock (B and D). Both cell lines show an increase in the cytoplasmic amount of HSP72. HSP72 in CX+ cells is predominantly localized in vesicle-like cell compartments; in CX- cells HSP72 is localized cytoplasmically. The cell surface staining pattern remains unaltered in CX+ and CX- cells after heat shock. Filled arrows denote enrichment of HSP72 on cellular projections on the plasma membrane; open arrows mark vesicle-like structures; "N" indicates the nucleus; $\times 35,000$.

CX- ($p = 0.032$) cells that also exhibit HSP72 on the cell surface on ~10 to 20% of the cells.

Discussion

Besides their essential intracellular functions for the survival of cells under normal as well as under stressful conditions, HSP72 has been found to be involved in a number of pathologic conditions where it is regarded as highly immunodominant for the immune system of the host: HSP70 proteins play a role in autoimmune diseases (6, 29–31), in neurodegenerative diseases (32), in inflammatory processes in transplant rejection (6, 33), in virus (9, 31, 34, 35), and in parasitic infections (36–38). Furthermore, members of the HSP70 family elicit strong cellular immune responses against cancer (4, 16, 21, 39–43). In this study, we show that the sensitivity of tumor cells to lysis mediated by a subset of NK cells correlates to HSP72 cell surface expression. Previously, we have shown that heat stress results in cell surface expression of HSP72 on human sarcoma cells that increases the sensitivity to lysis mediated by NK cells (5, 21). Here we demonstrate within an "autologous" colon carcinoma cell system that cell surface expression of HSP72 is a relevant target structure for an enhanced sensitivity to lysis even under physiologic conditions. By electron microscopic analysis, we show that the capacity of colon carcinoma cells to express HSP72 on the plasma membrane associates with differential subcellular distribution patterns of HSP72.

Although heat shock proteins are involved in membrane transport of other proteins, little is known about the mechanisms for HSP72 transportation to the plasma membrane. Unlike other members of the heat shock protein families (Grp78 or Grp96), HSP72 contains no classic C-terminal KDEL sequence for endoplasmic reticulum (ER) retention nor does it contain any other known signal peptide sequences for transmembrane transport (15). A vesicular location for basic fibroblast growth factor and IL-1 β and their non-ER-Golgi release suggest a role for alternative cytoplasmic protein export mechanism (42–44). Experiments indicating that inhibitors of the ER-Golgi route (brefeldin A; colchicine) do not block cell surface expression of HSP72 have been interpreted as an evidence for a non-ER-Golgi pathway of HSP72 (42). It has been shown that the constitutive HSP73 a member of the HSP70 family is located inside lysosomal vesicles (45). Members of the HSP70 families may chaperone other proteins into exosomes and therefore may be incorporated into vesicles during this process. Our results derived from electron microscopic studies demonstrate an accumulation of HSP72 in vesicle-like structures predominantly on tumor cells that exhibit an HSP72 cell surface expression. According to these findings, one might speculate that HSP72 is transported to the plasma membrane via lysosomal vesicles on an alternative non-ER-Golgi route. However, anchorage of HSP72 to the plasma membrane still remains enigmatic. Since an enrichment of HSP72 is found on cellular projections of CX+ tumor cells, one might speculate that HSP72 is associated

FIGURE 7. A, Comparison of the sensitivity of CX+, CX2, and CX- carcinoma cells to lysis mediated by NK-enriched effector cells (3–6% CD3; 45–52% CD16; 42–50% CD56). Lysis of CX+ cells (open circles) was higher than that of CX2 cells (open squares) and that of CX- cells (open triangles). E:T ratios ranged from 5:1 to 40:1. Each data point represents the mean value of at least three independent experiments. The spontaneous release for each target cell was <20%. A statistical analysis of the differences in lysis of CX- and CX+ cells and of CX- and CX2 cells is shown in Table III. B, Comparative Ab inhibition assay of the lysis of CX+, CX2, and CX- cells using HSP72 mAb and an IgG1 isotype-matched control Ab. The lysis of CX+ and CX2 cells that express HSP72 on their cell surface was inhibited with the HSP72-specific mAb. No inhibition of lysis was observed with CX- cells. The isotype-matched control Ab had no inhibitory effect on the three cell lines. E:T ratios ranged from 5:1 to 40:1. Each data point represents the mean value of at least three independent experiments. The spontaneous release for each target cell was <20%. A statistical analysis of the correlation of HSP72 cell surface expression and the sensitivity to lysis is shown in Table IV.

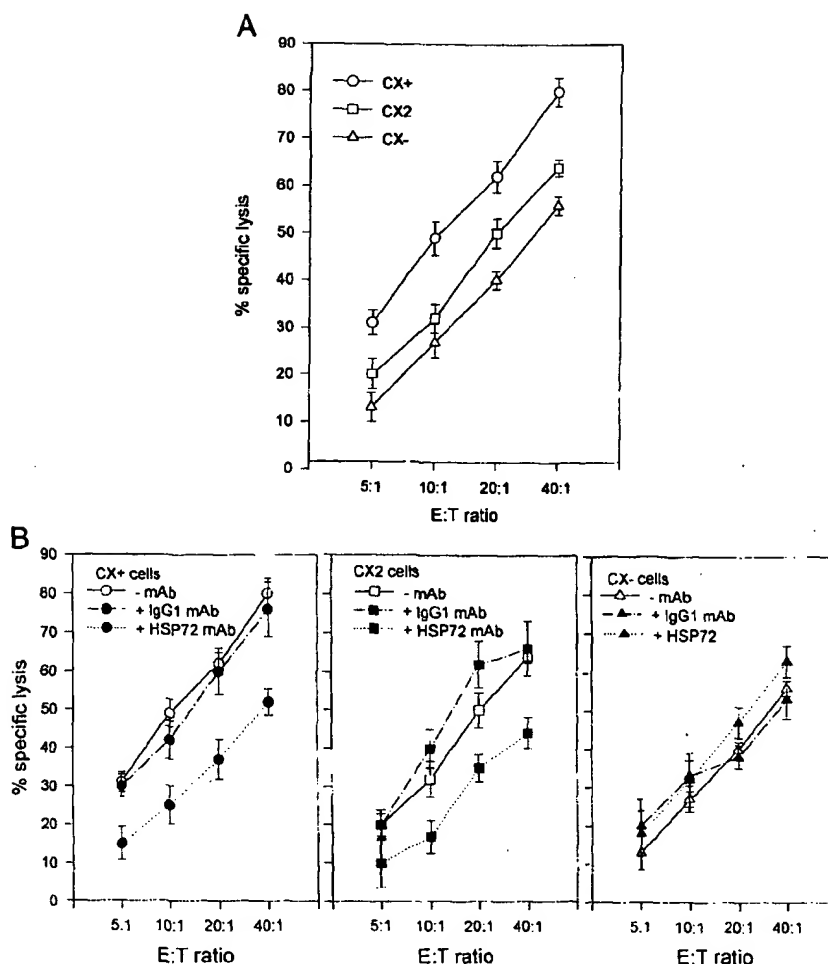


Table III. Calculation of the specific differences in the percentage in lysis of CX+, CX-, and CX2 cells^a

Ratio of Lysis of Tumor Cells	Mean Values	SD	p
CX+/CX-	1.794	0.425	0.033*
CX2/CX-	1.279	0.178	0.052

^a Data represent the means \pm SD ($n = 4$); * Significantly different from control levels ($p < 0.05$) by Student's *t* test.

with microfilaments in cell surface projections. By selective cell surface biotinylation of viable tumor cells, a 72-kDa protein was immunoprecipitated with HSP72-specific mAb, thus indicating that intact HSP72 is present in the plasma membrane.

The human colon carcinoma cell line CX2 exhibits an HSP72 cell surface expression on ~60% of the cells under physiologic conditions. In an effort to answer the question of whether a distinct cell cycle phase correlates to HSP72 cell surface expression, aphidicolin-synchronized CX2 colon carcinoma cells were used for simultaneous determination of the cell cycle distribution and the cell surface expression pattern. In contrast to the induction of HSP72 in normal rodent (43) and human cells (47, 48), the HSP72 expression in human colon carcinoma cells was not associated with a distinct phase of the cell cycle. This finding is in line with observations of Ferrarini et al. (4) who showed that tumor cells, in contrast to normal cells, lack a cell cycle-dependent expression of HSP72.

Table IV. Correlation of the percentage of specific lysis with the cell surface expression pattern of HSP72 on CX+, CX-, and CX2 cells^a

Ratio of Lysis and HSP72 Expression	Mean Values	SD	p
CX+/ctrl	0.935	0.053	0.094
CX+/HSP72	0.560	0.076	0.001*
CX2/ctrl	1.130	0.130	0.145
CX2/HSP72	0.604	0.103	0.005*
CX-/ctrl	1.164	0.280	0.327
CX-/HSP72	1.217	0.114	0.032*

^a Data represent the mean values of inhibition using an HSP72 (RPN1197, 3A3) and an isotype-matched IgG1 control (ctrl) antibody \pm SD ($n = 4$); * significantly different from control levels ($p < 0.05$) by Student's *t* test.

For further investigation of the possible role of HSP72 cell surface expression on colon carcinoma cells, a HSP72 high expressing (CX+) and a HSP72 low expressing (CX-) colon carcinoma subline were isolated from the CX2 cells. Despite the finding that directly after cell sorting no HSP72 cell surface expression was detectable on CX- cells, a stable amount of HSP72 expressing cells was present in all further cell passages. Since even after additional sorting experiments this low amount of CX+ cells (below 20%) was detectable in all further cell passages, we speculate that Ag capping is responsible for this phenomenon.

In contrast to the heat-inducible cell surface expression of HSP72 in sarcoma cells, heat stress did not alter the cell surface

expression pattern of CX+ and CX- cells, despite the fact that the cytoplasmic induction of HSP72 was comparable in all tumor cell types (5, 21). The electron microscopy data of untreated and heat-treated CX+ and CX- cells demonstrate that subcellular distribution rather than total amount determines the capacity to express HSP72 on the plasma membrane.

Members of the HSP70 family have been shown to elicit a cellular immune response both in vivo and in vitro (49). Here, the sensitivities to lysis of "autologous" colon carcinoma sublines that differ only in their capacity to express HSP72 on the cell surface were compared. Since our previous studies demonstrated that non-MHC-restricted large granular lymphocytes (21, 50) are relevant for the recognition of a stress-inducible, immunogenic HSP72 determinant, a NK-enriched effector cell population was used for the cytotoxicity studies on colon carcinoma sublines. The heat-independent HSP72 cell surface expression on CX2 and CX+ colon carcinoma cells strictly correlates with the sensitivity to lysis mediated by NK cells. These data indicate that besides HSP72 no additional heat-inducible factor is necessary for the recognition mechanism mediated by a HSP72-specific NK cell population. Presently, we cannot rule out whether tumor-specific peptides that are presented by HSP72 might play a role for NK recognition as proposed for T cell recognition of HSP70-associated peptides (15).

However, the role of MHC as an inhibitory signal for NK recognition as postulated by Moretta et al. (51) can be excluded for the HSP72-specific, adherent NK population for several reasons: 1) the lysis of CX+ and CX- carcinoma cells differed significantly despite the fact that the MHC alleles and the MHC expression level in both tumor sublines were identical; 2) by Ab-blocking studies no inhibitory effect on the lysis of both tumor sublines was observed by using the MHC class I-specific mAb W6/32 (28); 3) NK cells with specificity against HSP72 could be isolated from PBL of HLA-different human volunteers, thus indicating that the MHC background of the effector cell population does not influence the HSP72 specificity.

Decreased NK adhesion and killing induced by down-modulation of ICAM-1 expression (52) is unlikely since the killing of HSP72-expressing tumor cells did not correspond to cell density-dependent alterations in the expression levels of the classical cell adhesion molecule ICAM-1. The differences in expression of adhesion molecules on the tumor cells were only marginal and were insufficient to explain the differences in susceptibility to lysis and binding properties. Furthermore, coating of the target cells with Abs directed against ICAM-1 adhesion determinants had no effect on the lysis of susceptible tumor target cells (53, 54). Our results derived from Ab-blocking studies using ICAM-1 mAb on different carcinoma cell lines are in line with these findings (our unpublished observations). Besides negative signaling of NK recognition by certain MHC alloantigens (55) and positive recognition of carbohydrate-binding proteins via lectin receptors (56) on NK cells, one must consider HSP72 expression as an additional possible structure that determines the susceptibility of tumor target cells to lysis mediated by an NK subpopulation.

The "autologous" colon carcinoma cell system might provide new insights in explaining unusual cell surface localization of HSP72 on tumor cells. Comparative analysis of the membrane lipid composition, membrane fluidity, or measurement of intracellular pH in both tumor cell sublines might explain how HSP72 is anchored in the plasma membrane. Furthermore, these autologous tumor cells provide useful tools with which to study the role of cell surface expression of HSP72 in tumorigenicity and metastasis in animal models.

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References

- Welch, W. J. 1992. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol. Rev.* 72:1063.
- Craig, E. A., B. D. Gambill, and R. J. Nelson. 1993. Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol. Rev.* 57:402.
- Welch, W. J., and J. R. Feramisco. 1984. Nuclear and nucleolar localization of the 72,000-dalton heat shock protein in heat-shocked mammalian cells. *J. Biol. Chem.* 259:4501.
- Ferrari, M., S. Heltai, M. R. Zocchi, and C. Rugarli. 1992. Unusual expression and localization of heat-shock proteins in human tumor cells. *Int. J. Cancer* 51:613.
- Multhoff, G., C. Botzler, M. Wiesnet, E. Müller, T. Meier, W. Wilmanns, and R. D. Issels. 1995. A stress-inducible 72 kDa heat-shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. *Int. J. Cancer* 61:272.
- Lamb, J. R., V. Bal, J. B. Rothbard, A. Mehler, P. Mendez-Samperio, and D. B. Young. 1989. The mycobacterial GroEL stress protein: a common target of T-cell recognition in infection and autoimmunity. *J. Autoimmun.* 2(Suppl.):93.
- Heufelder, A. E., B. E. Wenzel, and R. S. Bahn. 1992. Cell surface localization of a 72 kilodalton heat shock protein in retinoblastic fibroblasts from patients with Graves' ophthalmopathy. *J. Clin. Endocrinol. Metab.* 74:732.
- Jacquier-Sarlin, M. R., K. Fuller, A. T. Dinh-Xuan, M. J. Richard, and B. S. Polla. 1994. Protective effects of hsp70 in inflammation. *Experientia* 50:1031.
- Polla, B. S., S. Kantengwa, G. J. Gleich, M. Kondo, C. M. Reimert, and A. F. Junod. 1993. Spontaneous heat shock protein synthesis by alveolar macrophages in interstitial lung disease associated with phagocytosis of eosinophils. *Eur. Respir. J.* 6:483.
- Ullrich, S. J., E. A. Robinson, L. W. Law, M. Willingham, and E. Appella. 1986. A mouse tumor-specific transplantation antigen is a heat shock-related protein. *Proc. Natl. Acad. Sci. USA* 83:3121.
- McNoret, A., Y. Patry, C. Burg, and J. Le-Pendu. 1995. Co-segregation of tumor immunogenicity with expression of inducible but not constitutive hsp70 in rat colon carcinomas. *J. Immunol.* 155:740.
- Srivastava, P. K., and M. R. Das. 1984. The serologically unique cell surface antigen of 72 kDa ascitic hepatoma is also its tumor-associated transplantation antigen. *Int. J. Cancer* 33:417.
- Blachere, N. E., H. Udono, S. Janetzki, Z. Li, M. Heike, and P. K. Srivastava. 1993. Heat shock protein vaccines against cancer. *J. Immunother.* 14:352.
- Udono, H., D. L. Levey, and P. K. Srivastava. 1994. Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8⁺ T cells in vivo. *Proc. Natl. Acad. Sci. USA* 91:3077.
- Udono, H., and P. K. Srivastava. 1994. Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J. Immunol.* 152:5398.
- Suto, R., and P. K. Srivastava. 1995. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 269:1585.
- Srivastava, P. K. 1994. Heat shock proteins in immune response to cancer: the Fourth Paradigm. *Experientia* 50:1054.
- Vigh, L., D. A. Los, I. Horvath, and N. Murata. 1993. The primary signal in the biological perception of temperature: Pd-catalyzed hydrogenation of membrane lipids stimulated the expression of the *desA* gene in *Synechocystis* PCC6803. *Proc. Natl. Acad. Sci. USA* 90:9090.
- Fujii, G., S. Horvath, S. Woodward, F. Eiserling, and D. Eisenberg. 1992. A molecular model for membrane fusion based on solution studies of an amphiphilic peptide from HIV gp41. *Protein Sci.* 1:1454.
- Vujanovic, N. L., H. Rabinovich, Y. J. Lee, R. B. Herberman, and T. L. Whiteside. 1993. Distinct phenotypic and functional characteristics of human natural killer cells obtained by rapid interleukin 2-induced adherence to plastic. *Cell. Immunol.* 151:133.
- Multhoff, G., C. Botzler, M. Wiesnet, G. Eissner, and R. Issels. 1995. CD3-large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72 kDa heat shock protein on human sarcoma cells. *Blood* 86:1374.
- Kung, P., G. Goldstein, E. L. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science* 206:347.
- Nicoletti, I., Miglierati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide and flow cytometry. *J. Immunol. Methods* 139:271.
- Danscher, G. 1981. Localization of gold in biological tissue: a photochemical method for light and electron microscopy. *Histochemistry* 71:81.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350.
- MacDonald, H. R., H. D. Engers, J. C. Cerotini, and K. T. Brunner. 1974. Generation of cytotoxic T lymphocytes in vitro. *J. Exp. Med.* 140:718.

28. Parham, P., M. J. Androlewicz, F. M. Brodsky, N. J. Holmes, and J. P. Ways. 1982. Monoclonal antibodies: purification, fragmentation and application to structural and functional studies of class I MHC antigens. *J. Immunol. Methods* 53:133.
29. Winfield, J. B. 1989. Stress proteins, arthritis and autoimmunity. *Arthritis Rheum.* 31:1221.
30. Weiss, R. A., M. P. Marfais, J. F. Tomaszewski, and C. J. Kelly. 1994. T cells reactive to an inducible heat shock protein induce disease in toxin-induced interstitial nephritis. *J. Exp. Med.* 180:2239.
31. de Gracff-Meeder, E. R., W. van-Eden, G. T. Rijkers, B. J. Prakken, B. J. Zegers, and W. Kuis. 1993. Heat-shock proteins and juvenile chronic arthritis. *Clin. Exp. Rheumatol.* 11(Suppl. 9):S25.
32. Chopp, M. 1993. The roles of heat shock proteins and immediate early genes in central nervous system normal function and pathology. *Curr. Opin. Neurol. Neurosurg.* 6:6.
33. Moliterno, R., L. Valdivia, F. Pan, and R. J. Duquesnoy. 1995. Heat shock protein reactivity of lymphocytes isolated from heterotopic rat cardiac allografts. *Transplantation* 59:598.
34. Poccia, F., P. Piselli, S. Di-Cesare, S. Bach, V. Colizzi, M. Mattei, A. Bolognesi, and F. Stirpe. 1992. Recognition and killing of tumour cells expressing heat shock protein 65 kDa with immunotoxins containing saporin. *Br. J. Cancer* 66:427.
35. Di-Cesare, S., F. Poccia, A. Mastino, and V. Colizzi. 1992. Surface expressed heat-shock proteins by stressed or human immunodeficiency virus (HIV)-infected lymphoid cells represent the target for antibody-dependent cellular cytotoxicity. *Immunology* 76:341.
36. Polla, B. S. 1991. Heat shock proteins in host-parasite interactions. *Immunol. Today* 12:38.
37. Shinnick, T. M. 1991. Heat shock proteins as antigens of bacterial and parasitic pathogens. *Curr. Top. Microbiol. Immunol.* 167:145.
38. Engman, D. M., E. A. Dragon, and J. E. Donelson. 1990. Human humoral immunity to hsp70 during *Trypanosoma cruzi* infection. *J. Immunol.* 144:3987.
39. Ferrarini, M., S. M. Pupa, M. R. Zocchi, C. Rgarli, and S. Menard. 1994. Distribution of HSP72 and monomeric laminin receptor expression in human lung cancers infiltrated by gamma delta T lymphocytes. *Int. J. Cancer* 57:486.
40. Tamura, Y., N. Tsuboi, N. Sato, and K. Kikuchi. 1993. 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. *J. Immunol.* 151:5516.
41. Tsuboi, N., M. Ishikawa, Y. Tamura, S. Takayama, H. Tobioaka, A. Matsuura, K. Hirayoshi, K. Nagata, N. Sato, and K. Kikuchi. 1994. Monoclonal antibody specifically reacting against 73-kilodalton heat shock cognate protein: possible expression on mammalian cell surface. *Hybridoma* 13:373.
42. Multhoff, G., and L. Hightower. 1996. Cell surface expression of heat shock proteins and the immune response. *Cell Stress Chaperones* 1:167.
43. Chouchane, L., S. Bowers, S. Sawasdikosol, R. M. Simpson, and T. J. Kindt. 1994. Heat shock proteins expressed on the surface of human T cell leukemia virus type I-infected cell lines induce autoantibodies in rabbits. *J. Infect. Dis.* 169:253.
44. Mignatti, P., T. Morimoto, and D. B. Rifkin. 1992. Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *J. Cell. Physiol.* 151:81.
45. Rubartelli, A., F. Cozzolino, M. Talio, and R. Sitia. 1990. A novel secretory pathway for interleukin-1 β , a protein lacking a signal sequence. *EMBO J.* 9:1503.
46. Terleckey, S. R. 1994. HSP70s and lysosomal proteolysis. *Experientia* 50:1021.
47. Hang, H., and M. H. Fox. 1995. Expression of hsp70 induced in CHO cells by 45.0 degrees C hyperthermia is cell cycle associated and DNA synthesis dependent. *Cytometry* 19:119.
48. Milarski, K. L., W. J. Welch, and R. I. Morimoto. 1989. Cell cycle-dependent association of HSP70 with specific cellular proteins. *J. Cell Biol.* 108:413.
49. Gomez, F. J., A. M. Gomez, and G. S. J. Deepe. 1992. An 80-kilodalton antigen from *Histoplasma capsulatum* that has homology to heat shock protein 70 induces cell-mediated immune responses and protection in mice. *Infect. Immun.* 60:2565.
50. Botzler, C., H. J. Kolb, R. D. Issels, and G. Multhoff. 1996. Nontoxic alkyl-lysophospholipid treatment increases sensitivity of leukemic K562 cells to lysis by natural killer (NK) cells. *Int. J. Cancer* 65:633.
51. Moretta, L., E. Ciccone, A. Poggi, M. C. Mingari, and A. Moretta. 1994. Ontogeny, specific functions and receptors of human natural killer cells. *Immunol. Lett.* 40:83.
52. Zamai, L., G. Zauli, A. Bavelloni, S. Marmioli, A. Cataldi, G. Weber, and M. Vitale. 1995. Tiazofurin induces a down-modulation of ICAM-1 expression on K562 target cells impairing NK adhesion and killing. *Cell. Immunol.* 164:100.
53. Quillet-Mary, A., L. Cavarec, N. Kermaec, C. Marchiol-Fourmignault, M. L. Gil, H. Conjeaud, and D. Fradelizi. 1991. Target lysis by human LAK cells is critically dependent upon target binding properties, but LFA-1, LFA-3 and ICAM-1 are not the major adhesion ligands on targets. *Int. J. Cancer* 47:473.
54. Akella, R., and R. E. Hall. 1992. Expression of the adhesion molecules ICAM-1 and ICAM-2 on tumor cell lines does not correlate with their susceptibility to natural killer cell-mediated cytotoxicity: evidence for additional ligands for effector cell beta integrins. *Eur. J. Immunol.* 22:1069.
55. Vitale, M., S. Sileri, D. Pende, L. Moretta, and A. Moretta. 1995. Coexpression of two functionally independent p58 inhibitory receptors in human natural killer cell clones results in the inability to kill all normal allogeneic target cells. *Proc. Natl. Acad. Sci. USA* 92:3536.
56. Brennan, J., F. Takei, S. Wong, and D. L. Mager. 1995. Carbohydrate recognition by a natural killer cell receptor, Ly-49C. *J. Biol. Chem.* 270:9691.

NATURAL KILLER CELL CLONES CAN EFFICIENTLY PROCESS AND PRESENT PROTEIN ANTIGENS¹

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NK cell clones obtained from three different donors were tested for their ability to present soluble proteins to Ag-specific T cell clones. All NK clones were CD2⁺CD3⁺CD56⁺, whereas the expression of CD16 varied from clone to clone. The NK cell clones were able to process and present tetanus toxoid (TT) to TT-specific T cell clones in a class II HLA restricted manner. The capacity of NK cell clones to function as APC was also observed using the house dust mite allergen *Der p* I and the *Der p* I-derived peptide Val89-Cys117. As with EBV-transformed B cell line, NK cell clones could present the peptide 3-13 derived from the 65-kDa heat shock protein of *Mycobacterium leprae*, but they were unable to present the whole *M. leprae* Ag. Freshly isolated NK cells, IL-2-activated NK cells, and NK cell lines expanded in vitro could also process and present TT. The ability of the different NK populations to act as accessory cells correlated with their levels of class II HLA expression. These data demonstrate that NK cell clones can efficiently function as APC, however they may be restricted in the types of Ag that they can process.

Activation of CD4⁺ Th cells by soluble protein Ag requires recognition of Ag in association with class II HLA molecules on the surface of APC. Since most soluble Ag do not bind in their native form to class II HLA molecules, APC have to capture these Ag, internalize and process them into short peptides able to bind to class II HLA molecules, and then re-expose the Ag-class II HLA complexes on the cell surface (1). Several studies have been performed to investigate the mechanism by which accessory cells uptake, process, and present Ag to T cells (1, 2). Recently, it has become clear that the efficiency of Ag uptake and the levels of biosynthesis and expression of the class II HLA molecules determine to a large extent the Ag-presenting capacity of a given cell (2, 3). In addition, cytokines produced by the APC contribute to Ag-induced T cell activation (4, 5). Besides monocytes and macrophages, other cell types, such as B lymphocytes and Langerhans cells/dendritic cells, have been demon-

strated to function as APC (5, 6).

NK cells are defined as LGL³ that do not express CD3 or any of the T cell receptor chains, but they are CD56 positive (7). Almost all of the NK cells isolated from normal donors are positive for the low affinity Fcγ receptor III (CD16). However, a minority of CD56⁺ NK cells fail to express CD16 or express CD16 at low levels (8). They are operationally defined as lymphoid cells that mediate non-HLA-restricted cytotoxicity against certain tumor and virus-infected targets spontaneously and without prior sensitization (9). Furthermore, NK cells exert a variety of functions, including regulatory functions on the adaptive immune system and on hematopoiesis and natural resistance against microbial infections, which are presumably mediated through secretion of cytokines (7-10). Recently the availability of NK cell clones provided a unique opportunity to study NK cell functions using homogeneous cell preparations purged of any contaminant cells.

The aim of the present study was to define whether highly purified NK cells and cloned NK cells can function as APC in HLA-restricted Ag-specific T cell responses. Our results demonstrated, at the clonal level, that activated NK cells can present soluble protein Ag to T cell clones. Their ability to internalize and process protein Ag was observed for both TT and *Der p* I, which is the major allergen in extracts of the house dust mite *Der pt*. These findings indicate that in addition to their capacity to mediate non-HLA-restricted lysis of tumor cells or virus infected targets, NK cells have the ability to process and present Ag to helper T cells.

MATERIALS AND METHODS

Isolation and purification of NK cells. Purification of NK cells from Ficoll-Hypaque isolated PBMC was performed using either a Percoll gradient or separation by magnetic beads. In the first procedure, PBMC were incubated for 4 h at 37°C on Petri dishes and then passed through a nylon wool column to remove monocytes and B cells. Nonadherent lymphocytes were fractionated by centrifugation on discontinuous gradients consisting of 30% and 40% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in PBS containing 10% FCS. Low buoyant density and high buoyant density lymphocytes were isolated from the interface and bottom of the Percoll gradients, respectively. The population of lymphocytes in the interface fraction was highly enriched for cells with LGL morphology. Staining of this LGL population with the Leu11 (anti-CD16) and Leu19 (anti-CD56) mAb revealed that between 30 and 50% Leu11⁺Leu19⁺ cells were present. Leu19⁺ cells were sorted with a FACStar Plus (Becton-Dickinson). The purity of these cells was greater than 99.5% upon reanalysis. Contamination of these cells with monocytes was assessed with phycoerythrin-labeled anti-CD14 (Leu-M3; Becton-Dickinson) analyzed by FACScan (Becton-Dickinson) and found to be

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³ Abbreviations used in this paper: LGL, large granular lymphocyte; TT, tetanus toxoid; EBV-LCL, EBV-transformed B cell line; *Der pt*, *Dermatophagoides pteronyssinus*; hsp, heat shock protein.

below detection levels (<0.2%).

Before cloning, NK cells were purified by separation using magnetic beads. Briefly, 5 to 10×10^6 cells were washed in PBS containing 1% BSA and 0.1% NaN_3 for 10 min at $250 \times g$ at 4°C . The pellet was then incubated for 30 min at 4°C with Leu11 and Leu19 mAb at a concentration of $1 \mu\text{g}/10^6$ cells. After two washes in PBS/ NaN_3 /BSA, the cells were incubated with biotinylated goat anti-mouse mAb (Tago, Burlingame, CA), at $1 \mu\text{g}/10^6$ cells, for 30 min at 4°C . The cells were washed twice and the pellet was incubated with streptavidin-FITC for 15 min at 4°C . After two washes in PBS, the cells were resuspended in a solution containing magnetic particles (11) diluted 1:100 in PBS/ NaN_3 /BSA. After 5 min incubation at 4°C , the cells were loaded to an iron wool column and separated with the MACS (Miltenyi, Sunnyvale, CA), as described previously (11). The cells stained with Leu11 and Leu19 mAb were labeled with FITC avidin and were sorted to a purity of 99.5%.

Establishment and culture of NK cell clones. $\text{CD16}^+\text{CD56}^+$ cells were resuspended in Yssel's medium (12) containing 1% human serum and cloned by limiting dilution at a concentration of one to five cells/well in 96-well round bottomed plates (Titertek; Flow Laboratories, McLean, VA) in the presence of a feeder cell mixture consisting of 5×10^5 irradiated (4000 rad) allogeneic PBMC/ml, 5×10^4 irradiated (5000 rad) cells/ml of the allogeneic EBV-LCL JY, and $0.1 \mu\text{g}/\text{ml}$ purified PHA (Wellcome Diagnostics, Beckenham, United Kingdom). After 7 days, $100 \mu\text{l}$ of medium containing $20 \text{ IU}/\text{ml}$ rIL-2, kindly provided by Dr. R. Kastelein (DNAX Research Institute, Palo Alto, CA), was added to each well. After 12 to 14 days, proliferating cultures were transferred to 24-well tissue culture plates (Linbro; Flow Laboratories) and restimulated with the feeder cell mixture. At day 5, the NK clones were washed and further expanded in medium containing rIL-2 ($20 \text{ U}/\text{ml}$). At day 8 or 9, they were restimulated with the feeder cell mixture. Six to 8 days after the last stimulation, the clones were screened in functional assays.

Cytotoxic assays. Cytotoxic activity was determined using a ^{51}Cr -release assay. Effector cells were mixed with 2×10^3 ^{51}Cr -labeled target cells in $200 \mu\text{l}$ Iscove medium (Gibco, Glasgow, United Kingdom) with 0.25% BSA in U-shaped wells of a microtiter plate, spun down at $50 \times g$, and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 . The supernatants were harvested using a Skatron supernatant collection system (Skatron, Lier, Norway) and were counted in a γ -counter. The maximum release was determined after incubation of the cells in 1% Triton X-100 and the spontaneous release by measuring the release of target cells in medium only. The percentage of specific ^{51}Cr release was determined as follows:

$$^{51}\text{Cr release} = 100 \times \frac{[(\text{experimental release}) - (\text{spontaneous release})]}{[(\text{maximum release}) - (\text{spontaneous release})]}$$

The data are presented as the mean of triplicate measurements.

Preparation of APC. The NK clones used as APC were taken 6 to 8 days after they had been activated by feeder cells, irradiated at 4000 rad, and washed three times. Before they were added to the culture, the NK cell clones were labeled with anti-CD14 mAb Leu M3 to look for possible contamination by residual feeder cells. No detectable CD14^+ cells were observed in these preparations. The EBV-LCL and fresh PBMC, used as APC, were irradiated 5000 and 4000 rad, respectively, and washed twice.

To test the APC function of freshly isolated NK cells, three sources of irradiated NK cells were used: purified NK cells directly isolated from PBMC as described above, purified NK cells cultured in vitro for 5 days in the presence of $200 \text{ IU}/\text{ml}$ of IL-2, and purified NK cell lines tested at day 5 after activation with the feeder cell mixture described above. These three different types of NK cells were preincubated overnight in the absence or in the presence of $10 \mu\text{g}/\text{ml}$ of TT in 5% CO_2 at 37°C . NK cells preincubated in medium only and Ag-pulsed cells were then irradiated, washed three times, resuspended in medium, and used as APC for the proliferative responses of TT specific T cell clones. For chloroquine treatment, the APC were suspended at $10^6/\text{ml}$ in Yssel's medium, and freshly prepared chloroquine (Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 0.1 mM , 30 min before the addition of TT at a concentration of $5 \mu\text{g}/\text{ml}$. After 5 h, the cells were washed three times with PBS and fixed with 0.05% glutaraldehyde for 1 min at room temperature. The reaction was stopped with 0.2 M lysine in PBS. The cells were washed three times and resuspended in medium.

Cloned Ag-specific T lymphocytes. The TT-specific T cell clones 827 and SP-F1, SP-F9, SP-F11, SP-F15, SP-F17, SP-F3, and SP-F14 have been obtained from the peripheral blood of a normal donor and a severe combined immunodeficient patient reconstituted with fetal liver and thymus transplantation, respectively, as described previously (12). The proliferative response of T cell clone 827 is restricted by HLA-DR3. Clones SP-F1 and SP-F11 are restricted by HLA-DR4, whereas clones SP-F9, SP-F15, and SP-F17 recognize TT in the context of HLA-DRw11. Clones SP-F3 and SP-F14 are promiscuous and recognize Ag processed and presented by any EBV-LCL, regard-

TABLE I
Phenotype and cytotoxic activity of NK clones

NK Clone	Phenotype ^a	Target Cells (% Cr release ^b)			
		K562	Jurkat	Daudi	F17
MK36	$\text{CD16}^{\text{bright}}$	65	38	22	4
MK42	CD16^{dim}	50	40	25	2
MK81	CD16^-	10	7	3	0
LR111	CD16^{dim}	40	33	30	3
LR115	$\text{CD16}^{\text{bright}}$	48	36	29	4
NPK1	$\text{CD16}^{\text{bright}}$	53	48	45	1

^a All the clones were $\text{CD2}^+\text{CD3}^+\text{CD56}^+$.

^b E:T cell ratio = 1:1.

less of its HLA phenotype (12). The cloned T cell lines, NP-12 and NP-14, specific for group I allergens of *Der pt* were generated from the blood of a patient allergic to *Der pt* by stimulating his PBMC with $1 \mu\text{g}/\text{ml}$ of a lyophilized extract of semi-purified *Der pt*⁴ (Diephuis Laboratories, Groningen, The Netherlands). These clones react with the recombinant *Der p* 1 molecule and with the *Der p* 1-derived peptide Val89-Cys117 (VQESYRYVAREQSCRRPNAQR-FGISNYC). The T cell clone, RP1511, specific for *Mycobacterium leprae*, was obtained from a patient suffering from tuberculous leprosy, as described elsewhere (13). This clone recognizes a peptide sequence on the N terminus of the 65-kDa hsp (amino acids 3 to 13; KTIAY-DEEARR), which is an immunodominant epitope for HLA-DR3-restricted, *M. leprae* hsp 65-reactive cells.

Ag-induced T cell proliferation. Nine to 12 days after the last stimulation with the feeder cells mixture, the cloned T cells were washed three times, and 2×10^4 of these cells were incubated with 2×10^4 irradiated (5000 rad) APC in the presence or absence of soluble TT at a final dilution of $5 \mu\text{g}/\text{ml}$; the *Der pt* Ag or the *Der p* 1-derived peptide Val89-Cys117 at a concentration of $1 \mu\text{g}/\text{ml}$; the *M. leprae* or the *M. leprae* hsp 65-derived peptide aa. 3 to 13, in a final volume of $200 \mu\text{l}$ Yssel's medium with 1% human AB⁺ serum. After 3 days of incubation, $1 \mu\text{Ci}$ [^3H]TdR (New England Nuclear, Dreieich, Federal Republic of Germany) was added to each well. Four hours later, the cells were harvested onto glass fiber strips using a semi-automated cell harvester, and the amount of incorporated [^3H]TdR was assessed by liquid scintillation counting. The results are expressed as the mean of triplicate cultures \pm SD. The effect of mAb on the proliferative capacity of T cell clones was determined by adding varying amounts of mAb at the onset of the cultures. The following mAb were used as ascites fluid: W6/32, which detects a common determinant on class I HLA molecules (Sera Lab, Crowley Down, United Kingdom); mAb SPV-L3, which reacts with a monomorphic determinant on HLA-DQ molecules (14); and mAb Q5/13, which detects a determinant common to HLA-DR and HLA-DP molecules (a kind gift from Dr. S. Ferrone, Medical College, Valhalla, NY) (15).

Fluorescence analysis. One hundred thousand cells were added per well of a V-bottomed microtiter plate and washed once with PBS containing 0.02 mM NaN_3 and 1% BSA. The cells were then incubated with the following mAb: the anti-CD2 mAb CLB-11 (kindly provided by Dr. R. Van Lier, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, The Netherlands), the anti-CD3 mAb Leu4, the anti-CD56 mAb Leu19, the anti-CD16 mAb Leu11 (Becton-Dickinson), and the anti-HLA-DR, DP mAb Q5/13. After 30 min at 4°C , the cells were washed twice in PBS/ NaN_3 /BSA and incubated with a 1:40 dilution of FITC-labeled F(ab')₂ fragments of goat anti-mouse IgG (Tago, Burlingame, CA) for 30 min at 4°C . After three washes, the cells were analyzed using a FACScan (Becton-Dickinson). As a monocyte marker, the anti-CD14 mAb (Leu-M3, Becton-Dickinson), directly labeled with phycoerythrin, was used.

RESULTS

Phenotype and cytotoxic activity of NK cell clones. Cloned NK cell lines were isolated from three different donors. Their phenotypic characterization and cytolytic activity are shown in Table I. All NK clones were $\text{CD2}^+\text{CD3}^+\text{CD56}^+$ but differed in their CD16 expression. The NK clones MK36, LR115, and NPK1 were $\text{CD16}^{\text{bright}}$, clones MK42 and LR111 were CD16^{dim} , whereas clone MK81 did not express detectable levels of CD16. All NK

⁴ Yssel, H., H. Gascan, P. Schneider, H. Spits and J. E. de Vries. 1991. Excessive IL-4 production by allergen specific T cell clones from atopic patients is responsible for induction of IgE synthesis. Submitted for publication.

Figure 1. Proliferative responses of TT-specific CD4⁺ T cell clones SP-F1 and SP-F11, which are restricted by HLA-DR4; SP-F15 and SP-F17, which are restricted by HLA-DRw11; and 827, which is restricted by HLA-DR3, to TT (5 μ g/ml) presented either by the NK clones MK36, MK42, MK81, LR115, LR111, and NPK1 or by the autologous EBV-LCL SPS and HSY.

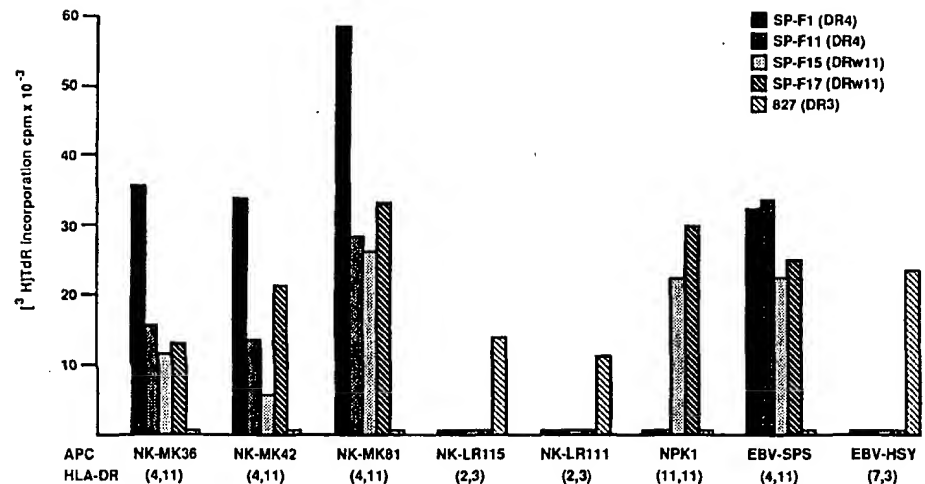


TABLE II

Effect of different Ag concentrations presented by NK cell clones on the proliferative responses of TT-specific T cell clones

Responder T Cell Clones	[TT] (μ g/ml)	APC ^a ([³ H]TdT incorporation cpm $\times 10^{-3}$)				
		Medium	MK36 ^b	MK42 ^b	MK81 ^b	SPS ^b
F1	15	2.6	40.6	38.3	42.6	58.0
	3	0.5	33.2	34.1	35.0	61.0
	0.6	0.2	15.1	17.4	18.2	55.3
	0.12	0.3	2.9	3.4	4.4	44.4
	0.024	0.1	1.9	1.5	1.9	20.7
	0.0048	0.1	0.7	1.1	1.0	0.6
	Medium	0.1	0.6	1.3	0.4	0.2
F14	15	1.3	8.3	8.2	11.3	4.4
	3	2.9	10.0	9.0	14.6	12.6
	0.6	3.8	10.4	10.1	16.0	20.8
	0.12	1.4	11.5	11.3	20.5	26.6
	0.024	0.1	4.8	5.3	15.0	22.9
	0.0048	0.1	1.0	1.2	2.2	4.8
	Medium	0.1	0.4	0.2	0.2	0.2

^a APC:responder ratio = 1:1.

^b The NK cell clones MK36, MK42, and MK81, and the EBV-LCL SPS were isolated from the same donor and are HLA-DR4,w11.

TABLE III

Effect of anti-HLA mAb on the proliferative response of T cell clone SP-F17 to TT^a

mAb	mAb ^b dilution	APC (% inhibition of proliferation)		
		SPS ^c	MK81 ^d	MK42 ^d
W6/32 (anti-HLA-A,B,C)	1:200	4	15	5
	1:600	0	12	0
	1:1800	0	15	0
SPV-L3 (anti-HLA-DQ)	1:200	2	16	1
	1:600	0	0	0
	1:1800	0	0	0
Q5/13 (anti-HLA-DP,DR)	1:200	80	75	81
	1:600	68	70	57
	1:1800	45	48	31

^a The proliferative response of F17 to TT (5 μ g/ml) in the absence of mAb was 28.9 ± 2.1 with SPS, 11.6 ± 1.0 with MK81, and 34.5 ± 2.7 with MK42 as APC (cpm $\times 10^{-3}$).

^b mAb were used as ascites fluid.

^c SPS is an EBV cell line.

^d MK81 and MK42 are NK clones originated from the same donor as SPS.

clones expressed high levels of class II HLA Ag as shown for clone MK36 (see Figure 4F). All the NK clones were highly cytotoxic for the NK-sensitive target cells K562, Daudi, and Jurkat at E:T ratios of 1:1 (Table I) or 10:1 (data not shown), except for MK81, which only killed these NK-sensitive target cells efficiently at E:T ratios of 10:1 (data not shown). On the other hand, all NK clones had no or very low cytotoxic activity against CD4⁺ TT-

TABLE IV

Ag processing by NK cell clones

APC	Treatment before Fixation	Responder T Cell Clones ([³ H] TdT incorporation cpm $\times 10^{-3a}$)			
		F3	F11	F17	F9
Medium	Medium	0.3	0.1	0.2	0.1
	Medium	0.1	0.1	0.2	0.1
MK36	TT ^b	19.8	2.0	15.0	8.9
	TT + chloroquine	9.8	0.5	0.7	0.1
MK36 (TT) ^c	Chloroquine	32.6	19.7	17.6	ND
	Medium	ND	0.4	0.8	ND
MK81	TT	ND	18.9	22.3	ND
	TT + chloroquine	ND	1.0	4.6	ND

^a In all cases, the SD was <10% of the total cpm.

^b TT: 5 μ g/ml.

^c MK36 preincubated overnight with TT, washed, and then put in the presence of chloroquine.

specific T cell clone F17, which was included as a control, non-NK-sensitive, target cell.

NK cell clones can present TT to CD4⁺ T cell clones. To determine whether NK cell clones could process and present Ag, NK clones obtained from three different donors were tested for their capacity to present TT to CD4⁺ TT-specific T cell clones. In Figure 1, it is shown that cloned NK cells can present TT to the T cell clones SP-F1, SP-F11, SP-F15, SP-F17, and 827 in a class II HLA-restricted fashion. The cloned TT-specific T cell line SP-F1 and SP-F11 are restricted by HLA-DR4, whereas the T cell clones SP-F15 and SP-F17 recognize the Ag in the context of HLA-DRw11. All four of these clones proliferated in response to TT, presented by the DR4,w11-positive NK clones MK36, MK42, and MK81. Furthermore, the HLA-DRw11-restricted T cell clones SP-F15 and SP-F17 proliferated also in response to TT presented by the NK cell clone NPK1, which is homozygous for HLA-DRw11. The HLA-DR3-restricted T cell clone 827 recognized TT presented by the NK clones LR115 and LR111 that express HLA-DR2,3. The proliferative responses of the CD4⁺ T cell clones to optimal concentrations of TT (5 μ g/ml) presented by the NK clones were in the same range as those obtained when the class II HLA-matched EBV-LCL, SPS and HSY (Fig. 1), or PBMC (data not shown) were used as APC. This was also the case when the APC, either NK cells or the EBV-LCL, were used at suboptimal concentrations (less than 1:1 ratio; data not shown). However, at suboptimal concentrations of Ag, EBV-LCL were more efficient APC than the NK clones (Table II). At

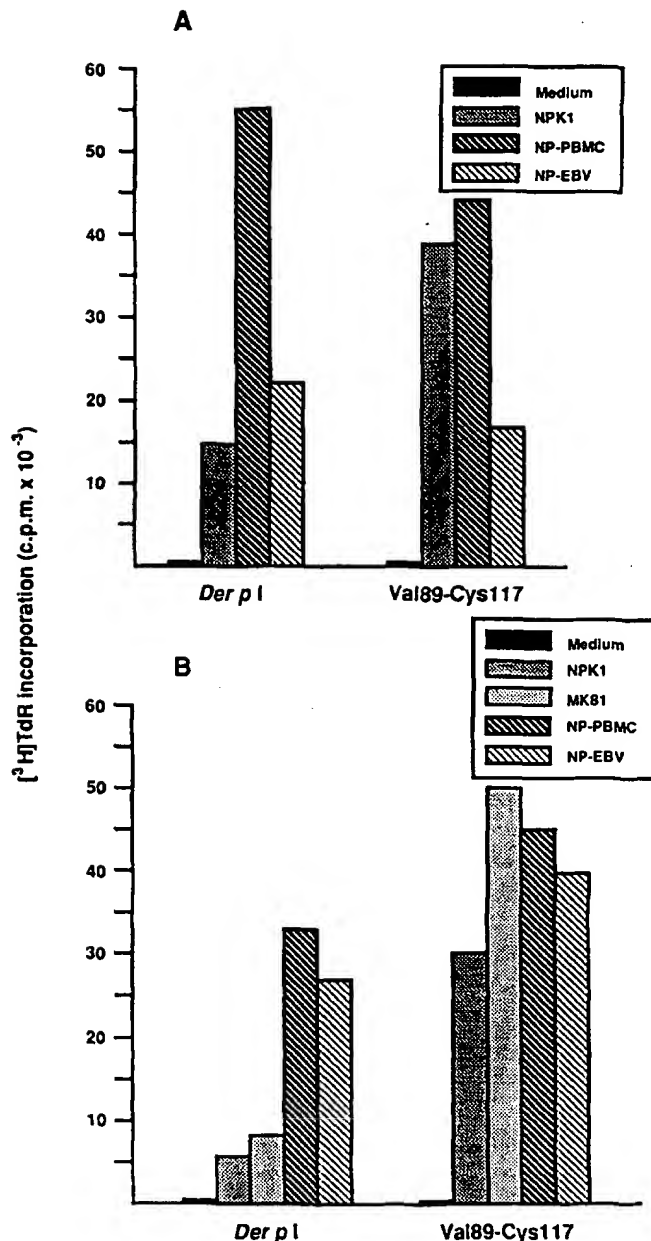


Figure 2. Proliferative responses of house dust mite-specific T cell clones NP-12 (A) and NP-44 (B) to *Der p I* Ag and to *Der p I*-derived peptide Val89-Cys117. As APC, the autologous NK cell clone NPK1, HLA-DRw11 matched NK cell clone MK81, autologous PBMC, and EBV-LCL were used.

concentrations of TT as low as 0.02 $\mu\text{g/ml}$, EBV-LCL were still able to induce moderate proliferative responses, whereas those induced by the NK cell clones were in general very low.

The notion that the TT-specific proliferative responses of the T cell clones were restricted by the HLA-DR Ag of the NK cell clones used as APC was confirmed by blocking studies with mAb against class II HLA Ag. In Table III, it is shown that the proliferative responses of T cell clone SP-F17 to TT, presented by the NK clones MK81 and MK42, were blocked by the mAb Q5/13, which is specific for HLA-DP,DR, but not by the mAb SPV-L3 and W6/32, which are directed against the HLA-DQ and the HLA class I Ag, respectively. Comparable results were found with the other TT-specific T cell clones (data not shown).

To exclude the possibility that the T cell clones recognize TT peptides, which could be present as contami-

nants in the TT preparation, the NK clones MK81 and MK36 were pulsed with TT in the presence or absence of chloroquine, which inhibits intracellular Ag processing (16). The cells were then fixed with glutaraldehyde to prevent any further processing and used as APC. As additional control, NK clone MK36 was incubated overnight with TT, washed, and then cultured in the presence of chloroquine plus responder T cells. In this case, chloroquine should not have any inhibitory effect because it was added to the culture after the uptake and processing of the Ag by the NK-APC. In Table IV, it is shown that the NK cell clones pulsed with TT and then fixed with glutaraldehyde can present Ag to the T cell clones SP-F3, SP-F11, SP-F17, and SP-F9. The addition of chloroquine during the Ag pulse resulted in strong reduction of proliferation of all four T cell clones tested. Treatment of MK36 cells with chloroquine, after the cells had been preincubated overnight with TT but before fixation, did not affect the proliferative responses of the T cell clone, indicating that chloroquine had no toxic effects. These data demonstrate that NK cell clones can internalize and process soluble protein Ag such as TT and express it on the membrane in form of a short peptide.

NK cell clones present the major house dust mite allergen *Der p I* to *Der p I*-specific T cell clones. The capacity of NK clones to present soluble proteins to Ag-specific T cell clones is not restricted to TT. In Figure 2, it is shown that the NK cell clone MK81, which is HLA-DRw11-compatible with donor NP, and the autologous NK clone NPK1, effectively present recombinant *Der p I* to the *Der p I* specific CD4⁺ T cell clones NP-12 and NP-44. In addition, NP-12 and NP-44 proliferated in response to the *Der p I*-derived peptide Val89-Cys117 presented by the NK clones. The levels of proliferation towards *Der p I* or Val89-Cys117 peptide presented by the NK clones were lower or comparable, respectively, to those obtained when the autologous EBV-transformed B cell line NP-EBV or PBMC of donor NP were used as APC.

NK cell clones fail to present whole *M. leprae*, but can present effectively the *M. leprae* hsp 65-derived peptide aa. 3-13. NK cell clones LR111 and LR115 were very inefficient in presenting the *M. leprae* to Ag-specific CD4⁺ T cell clones as compared with PBMC (Fig. 3A). This failure is not unique for NK cells, since the autologous EBV-transformed B cell lines were also inefficient APC for *M. leprae*. However, when very high concentrations (100 $\mu\text{g/ml}$) of Ag were used, low responses could be induced even using NK clones or EBV-LCL as APC. In contrast, PBMC were effective in presenting the whole *M. leprae* Ag to the specific CD4⁺ T cells. The T cell proliferation was dependent on the Ag concentrations. It has been reported previously that the monocytes present in the PBMC are responsible for uptake, processing, and presentation of *M. leprae* (17). The defective presentation of *M. leprae* by both NK cell clones and EBV-LCL is not due to their inability to present the relevant antigenic peptide, because both cell types could effectively present the 65-kDa-derived peptide 3-13 to the *M. leprae*-specific T cell clones (Fig. 3B). It is therefore likely that NK cell clones and EBV-LCL, in contrast to monocytes, are unable to uptake or process the whole *M. leprae*. However, this failure is related to the characteristic of the Ag and is not due to an intrinsic inability of the NK clones to uptake or process soluble protein Ag, since they could process TT and *Der p I* (Figs. 1 and 2).

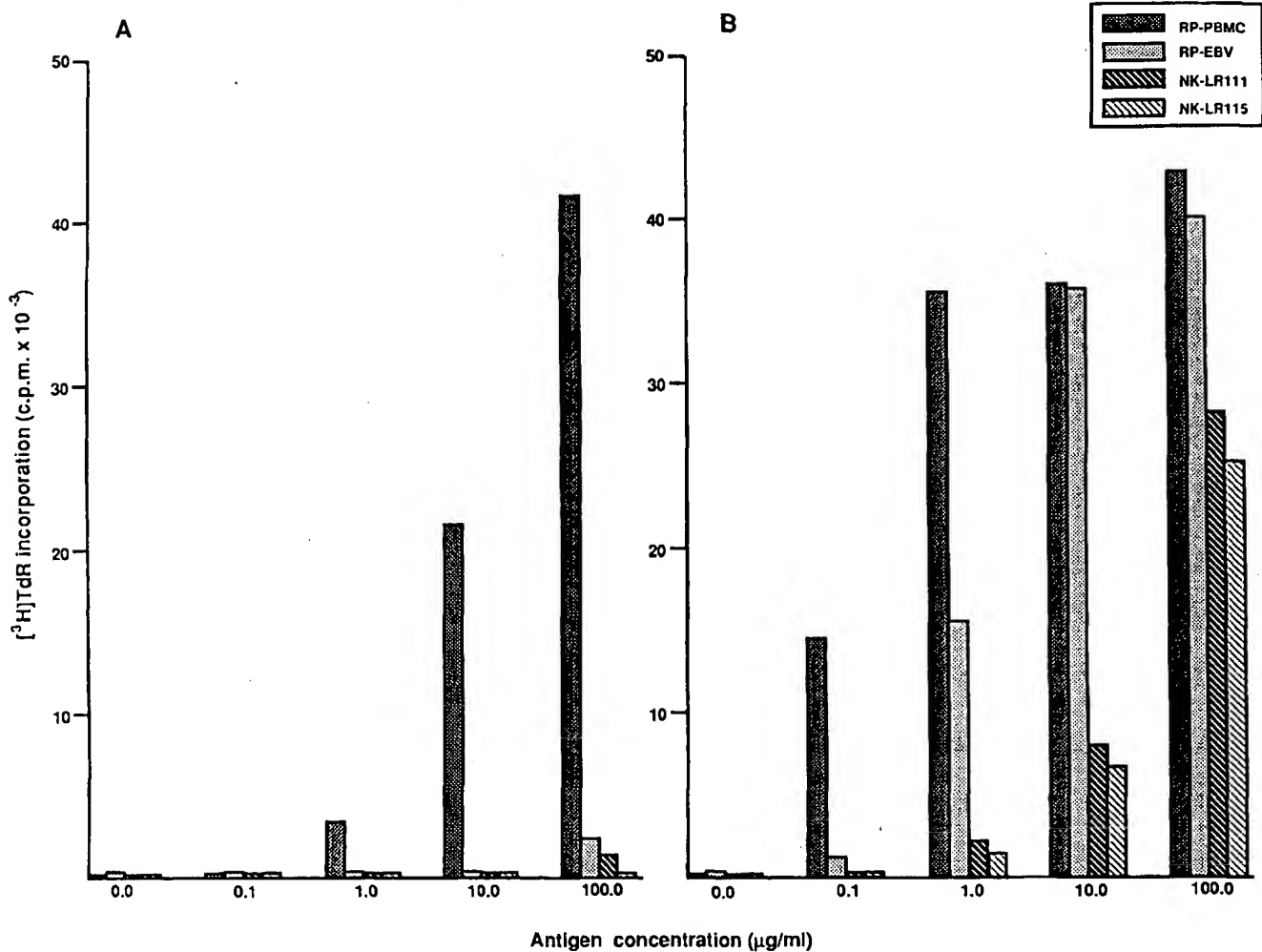


Figure 3. Proliferative responses of *M. leprae*-specific T cell clone RP-1511 to different concentrations of *M. leprae* (A) and *M. leprae*-derived peptide aa. 3-13 (B). As APC, the autologous NK clones NK-LR111 and NK-LR115, PBMC, and EBV-LCL were used.

TABLE V
Proliferative response of T cell clones to TT presented by fresh NK cells

APC ^a		Responder T Cell Clones ($[^3\text{H}]\text{TdR}$ incorporation cpm $\times 10^{-3}$)	
		F3	F14
SPS	1:1 ^b	36.7 ^c	18.9
	3:1	52.1	31.3
PBMC	1:1	27.8	11.2
	3:1	40.4	17.0
NK fresh	1:1	10.0	1.2
	3:1	16.5	5.7
NK + IL-2	1:1	38.0	18.1
	3:1	29.8	20.1
NK + feeder	1:1	52.8	37.2
	3:1	53.3	38.7
Medium		1.3	0.5

^a Preincubated overnight with 10 $\mu\text{g/ml}$ of TT. The proliferative responses of the T cell clones in the presence of non-Ag pulsed APC were, in every situation, comparable or less than those observed when these T cell clones were cultured in medium only (i.e., in the absence of APC).

^b APC:responder ratio.

^c Results are expressed as the mean cpm obtained in three different experiments performed on three different normal donors. In all cases, the SD was <10% of the total cpm.

APC function of freshly isolated NK cells. To investigate whether fresh NK cells can also process and present Ag, we isolated NK cells from peripheral blood of normal donors and tested their ability to present TT to the CD4⁺ T cell clones SP-F3 and SP-F14. These TT-specific clones

were used as responder cells because they have the particular property to recognize processed TT in association with any HLA-DR molecule (12). Therefore, their proliferative responses to TT can be tested using various HLA mismatched PBMC, EBV-LCL, or NK clones as APC.

As shown in Table V, fresh NK cells incubated overnight with TT have the capacity to process and present Ag. Their APC function is, however, less efficient than that of EBV-LCL or PBMC and varied from donor to donor (data not shown). The capacity to process and present TT was strongly enhanced when the NK cells were activated for 5 days with optimal concentrations of rIL-2 (200 U/ml) or with a mixture of feeder cells (as described in *Materials and Methods*). Proliferative responses of F3 and F14 were comparable when the EBV-LCL SPS, PBMC, or IL-2-activated NK cells, isolated from the same donor, were used. NK cell lines activated with feeder cells induced slightly higher T cell responses to TT. As shown in Figure 4, the different degrees of efficiency in Ag processing and presentation by freshly isolated NK, NK activated in vitro with IL-2, and NK cell lines, correlated with the different degrees of HLA class II expression by these cells.

DISCUSSION

NK cells are cytotoxic for virally infected cells and certain cancer cells and have therefore been implicated

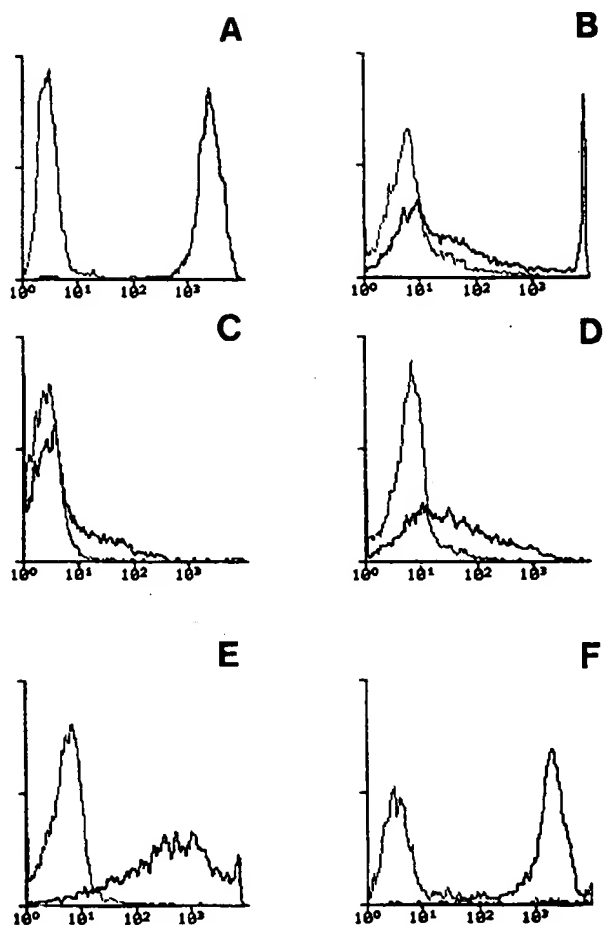


Figure 4. HLA-DR,DP expression on EBV-LCL (A), PBMC (B), freshly isolated NK cells (C), IL-2 activated NK cells (D), NK cell lines expanded with feeder mixture (E), and NK cell clone MK36 (F) was determined by FACS analysis using the G5/13 mAb.

to play a major role in first line immunodefense against malignancies and viral infections (7-9). In addition, NK cells have been reported to act as natural suppressor cells in graft-vs-host reactions after bone marrow transplantation (18). Furthermore, NK cells have important immunoregulatory functions that are mediated via cytokines produced by these cells (7-10).

In the present study, we demonstrate that highly purified activated NK cells and cloned NK process and present soluble Ag to CD4⁺ Ag-specific T cell clones. The ability of NK cell clones to present Ag was not due to contaminating monocytes or EBV-LCL from the feeder cell mixture, since we did not observe detectable CD14⁺ cells in our preparations. More importantly, Ag presentation was consistently restricted by the HLA-DR of the particular NK clone. In experiments like those presented in Figure 1, several clones with different HLA-DR phenotypes cultured with the same feeder cell mixture could only present Ag to those Ag-specific T cell clones restricted by the HLA-DR of the NK clones.

In addition, our data strongly suggest that freshly isolated NK cells can also present Ag. In this case it is, however, not possible to exclude that some monocytes were still present in the purified preparation, although our NK cells were rigorously depleted by a combination of adherence, nylon wool depletion, and sorting, and we could not detect any CD14⁺ cells in these NK populations. We show that activated NK cells are generally as effective

as APC as EBV-LCL and PBMC for both TT and the major allergen for house dust mite *Der p 1*. In addition, cloned NK cells were able to present a *Der p 1*-derived 29-mer peptide. The proliferative responses of TT and *Der p 1*-specific T cell clones towards these Ag presented by cloned NK cells were restricted by HLA-DR Ag and not by HLA-DQ Ag. NK cell clones pretreated with cloroquine failed to induce productive T cell proliferation in response to TT, indicating that TT presentation required processing of the Ag and was not mediated via TT-derived peptides, which could have been present in the TT preparations, accommodated in the HLA-DR molecules. Taken together, these data indicate that activated NK cells can efficiently process and present Ag in a HLA class II-restricted fashion to T cell clones and that in this aspect they are not different from other APC such as monocytes, macrophages, or EBV-LCL. However, it remains to be determined whether NK cells can activate resting T cells, as has been shown for macrophages and dendritic cells (1, 2). Interestingly, cloned NK cells, like EBV-LCL, were very inefficient in presenting the *M. leprae*, whereas monocytes have been shown to process and present this Ag efficiently (17). Nevertheless, both the cloned NK cells and the EBV-LCL were able to present the hsp 65-derived peptide aa. 3-13. Therefore, it may be concluded that the failure of NK cells or EBV-LCL to effectively present the *M. leprae* to *M. leprae*-specific CD4⁺ T cell clones is not due to a lack of binding of the immunodominant peptide to class II HLA molecules, but is probably related to the inability of these cells to appropriately uptake or process this Ag. Similar data have been described for Ag presentation of soluble bacterial streptolysin O by purified LGL isolated by cell sorter and completely depleted of both monocytes and dendritic cells (19). However, Scala et al. (20) reported that a subset of LGL can function as accessory cells for soluble streptolysin O. Individual T cell epitopes can have processing requirements that differ greatly, therefore it is possible that NK cells and EBV-LCL can modify and process only certain types of Ag.

It has been reported that biosynthesis and expression of class II HLA molecules are crucial factors to determine the Ag presenting capacity of a given cell (3, 21). Cloned NK cells express high levels of class II HLA Ag, which may account for their capacity to function as efficient APC. Freshly isolated NK cells that express very low levels of class II HLA were also capable of presenting TT to TT-specific T cell clones, although much less efficiently. The capacity of the freshly isolated NK cells to present Ag varied considerably from donor to donor, which may be due to the variable levels of HLA-DR molecules expressed on these cells. It is possible that a certain number of activated NK cells was present in some PBMC preparations obtained from normal donors. In addition, it is still controversial whether or not all the resting NK cells do not express the class II HLA Ag (19, 22). On the other hand, several cell types, including fibroblasts, epithelial and endothelial cells, as well as human-activated T cells, can express class II HLA molecules, but their role as APC has been debated because of failure to demonstrate presentation of soluble Ag (23-25). It has been reported that activated T cells and class II HLA thyroid epithelial cells can present denatured protein Ag or peptides to stimulate proliferation of Ag-specific T cell lines (25, 26), suggesting that the defect was at the level of Ag processing. An alternative possi-

bility, supported by recent work on activated T cells by Lanzavecchia et al. (27), is that these cells are limited in their ability to present conventional Ag such as TT by their inefficiency at Ag capture. Since NK cells have the capacity to present soluble proteins such as TT or *Der p* I, this implies that they can take up Ag nonspecifically. One possibility is that CD16 (Fc γ RIII) and/or related molecules, which are absent on most T cells, are involved in capturing Ag.

It is known that cytokines released by APC exert costimulatory activity in stimulating T cell proliferation. NK cell clones produce high levels of GM-CSF, IFN- γ , and IL-3 and express mRNA for TNF- α and IL-2 (H. Spits, unpublished data). In contrast, they fail to synthesize IL-1- β and IL-6, which are considered the most important cytokines in determining accessory functions of monocytes and transformed B cells (4, 5). The fact that activated NK cells produce cytokines that can act on T cells and that are not synthesized by monocytes or B cells, notably IFN- γ and IL-3, may imply that the Ag-specific responses of T cells is different, depending on the type of APC involved.

In conclusion, this study demonstrates that NK cells can efficiently present soluble Ag to T cell clones. It is conceivable that NK cells can also function as APC in vivo, and more effectively even in situations in which local IL-2 production occurs, which results in NK cell activation and HLA expression.

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REFERENCES

1. Unanue, E. R. 1984. Antigen-presenting function of the macrophage. *Annu. Rev. Immunol.* 2:395.
2. Lanzavecchia, A. 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* 8:773.
3. Puré, E., K. Inaba, M. T. Crowley, L. Tardelli, M. D. Witmer-Pack, G. Ruberti, G. Fathman, and R. M. Steinman. 1990. Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. *J. Exp. Med.* 172:1459.
4. Alello, F. B., D. L. Longo, R. Overton, L. Takacs, and S. K. Durum. 1990. A role for cytokines in antigen presentation: IL-1 and IL-4 induce accessory functions of antigen-presenting cells. *J. Immunol.* 144:2572.
5. O'Garra, A., G. Stapleton, V. Dhar, M. Pearce, J. Schumacher, H. Rugo, D. Barbis, A. Stall, J. Cupp, K. Moore, P. Vieira, T. Mosmann, A. Whitmore, L. Arnold, G. Houghton, and M. Howard. 1990. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Intern. Immunol.* 2:821.
6. Romani, N., S. Koide, M. Crowley, M. Witmer-Pack, A. M. Livingstone, C. G. Fathman, K. Inaba, and R. M. Steinman. 1989. Presentation of exogenous protein antigens by dendritic cells to T cell clones: intact protein is presented best by immature, epidermal Langerhans cells. *J. Exp. Med.* 169:1169.
7. Trinchieri, G. 1989. Biology of natural killer cells. In *Advances in Immunology*, Vol. 47. Academic Press, New York, p. 187.
8. Nagler, A., L. L. Lanier, S. Cwiria, and J. H. Phillips. 1989. Comparative studies of human FcRIII-positive and negative NK cells. *J. Immunol.* 143:3183.
9. Lanier, L. L., J. H. Phillips, J. Hackett, Jr., M. Tutt, and V. Kumar. 1986. Natural killer cells: definition of a cell type rather than a function. *J. Immunol.* 137:2735.
10. Young, H. A., and J. R. Ortaldo. 1987. One-signal requirement for interferon- γ production by human large granular lymphocytes. *J. Immunol.* 139:724.
11. Gascan, H., J.-F. Gauchat, M.-G. Roncarolo, H. Yssel, H. Spits, and J. E. de Vries. 1991. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by IL-4 and a signal provided by activated CD4 $^{+}$ T cell clones. *J. Exp. Med.* 173:747.
12. Roncarolo, M.-G., H. Yssel, J.-L. Touraine, R. Bacchetta, L. Gebuhrer, J. E. de Vries, and H. Spits. 1988. Antigen recognition by MHC-incompatible cells of a human mismatched chimera. *J. Exp. Med.* 168:2139.
13. Ottenhoff, T. H. M., and T. Mutis. 1990. Specific killing of cytotoxic T cells and antigen-presenting cells by CD4 $^{+}$ cytotoxic T cell clones: a novel potentially immunoregulatory T-T cell interaction. *J. Exp. Med.* 171:2011.
14. Spits, H., G. Keizer, J. Borst, C. Terhorst, A. Hekman, and J. E. de Vries. 1983. Characterization of monoclonal antibodies against cell surface molecules associated with the cytotoxic activity of natural and activated killer cells and cloned CTL lines. *Hybridoma* 2:423.
15. Quaranta, V., L. E. Walker, M. A. Pellegrino, and S. Ferrone. 1980. Purification of immunologically functional subsets of human Ia-like antigens on a monoclonal antibody (Q5/13) immunoadsorbent. *J. Immunol.* 125:1421.
16. Wibo, M., and B. Poole. 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B. *J. Cell. Biol.* 63:430.
17. Bhardwaj, V., and M. J. Colston. 1988. The processing and presentation of mycobacterial antigens by human monocytes. *Eur. J. Immunol.* 18:691.
18. Azuma, E., H. Yamamoto, and J. Kaplan. 1989. Use of lymphokine-activated killer cells to prevent bone marrow graft rejection and lethal graft-vs-host disease. *J. Immunol.* 143:1524.
19. Brooks, C. F., and M. Moore. 1986. Presentation of a soluble bacterial antigen and cell-surface alloantigens by large granular lymphocytes (LGL) in comparison with monocytes. *Immunology* 58:343.
20. Scala, G., P. Allavena, J. R. Ortaldo, R. B. Herberman, and J. J. Oppenheim. 1985. Subsets of human large granular lymphocytes (LGL) exhibit accessory cell functions. *J. Immunol.* 134:3049.
21. Peterson, M., and J. Miller. 1990. Invariant chain influences the immunological recognition of MHC class II molecules. *Nature (Lond.)* 345:172.
22. Perussia, B., and G. Trinchieri. 1984. Antibody 3G8, specific for the human neutrophil Fc receptor, reacts with natural killer cells. *J. Immunol.* 132:1410.
23. Shastri, N., B. Malissen, and L. Hood. 1985. Ia-transfected L-cell fibroblasts present a lysozyme peptide but not the native protein to lysozyme-specific T cells. *Proc. Natl. Acad. Sci. USA* 82:5885.
24. Lechler, R. I., M. A. Norcross, and R. N. Germain. 1985. Qualitative and quantitative studies of antigen-presenting cell function by using I-A expressing L cells. *J. Immunol.* 135:2914.
25. Gerrard, T. L., D. J. Volkman, C. H. Jurgensen, and A. S. Fauci. 1986. Activated human T cells can present denatured antigen. *Hum. Immunol.* 17:416.
26. Londei, M., J. R. Lamb, G. F. Bottazzo, and M. Feldman. 1984. Epithelial cells expressing aberrant MHC class II determinants can present antigen to cloned human T cells. *Nature (Lond.)* 312:639.
27. Lanzavecchia, A., E. Roosnek, T. Gregory, P. Berman, and S. Abrignani. 1988. T cells can present antigens such as HIV gp120 targeted to their own surface molecules. *Nature (Lond.)* 334:530.

CD3⁻ Large Granular Lymphocytes Recognize a Heat-Inducible Immunogenic Determinant Associated With the 72-kD Heat Shock Protein on Human Sarcoma Cells

By Gabriele Multhoff, Claus Botzler, Marion Wiesnet, Günther Eißner, and Rolf Issels

Traditionally, heat shock proteins (HSPs) are believed to be located intracellularly, where they perform a variety of chaperoning functions. Recently, evidence has accumulated that some tumor cells express HSPs on the cell surface. The present study confirms this finding and correlates HSP72 cell surface expression, induced by nonlethal heat shock, with an increased sensitivity to interleukin-2-stimulated CD3⁻ natural killer (NK) cells. After nonlethal heat shock, a monoclonal antibody directed against the major heat-inducible 72-kD HSP (HSP72) stains the cell surface of sarcoma cells (ie, Ewing's sarcoma cells or osteosarcoma cells) but not that of normal cells (ie, peripheral blood lymphocytes, fibroblasts, phytohemagglutinin-stimulated blasts, B-lymphoblastoid cell lines) or of mammary carcinoma cell line MX-1 carcinoma cells. In this study, we show for the first time a correlation of HSP72 cell surface expression with an increased susceptibility to lysis by NK effector cells. This finding is supported by the following points: (1) HLA-disparate effector cells show

similar, elevated lysis of HSP72⁺ heat-treated sarcoma cells; (2) CD3⁻ NK cells, but not CD3⁺ cytotoxic T lymphocytes, are responsible for the recognition of heat-shocked sarcoma cells; (3) by antibody-blocking studies, an immunogenic HSP72 determinant, which is expressed selectively on the cell surface of heat-treated sarcoma cells could be correlated with NK recognition; (4) the reported phenomenon is independent of a heat-induced, transient downregulation of major histocompatibility complex (MHC) class-I expression; and (5) blocking of MHC class-I-restricted recognition, using either MHC class-I-specific monoclonal antibody W6/32 on the target cells or α/β T-cell receptor monoclonal antibody WT31 on effector cells, also has no inhibitory effect on the lysis of HSP72⁺ tumor cells. Finally, our *in vitro* data might have further clinical implications with respect to HSP72 as a stress-inducible, sarcoma-specific NK recognition structure. © 1995 by The American Society of Hematology.

H EAT, LIKE OTHER STRESS factors, induces an increased synthesis of heat shock proteins (HSPs), especially of the highly stress-inducible 72-kD HSP (HSP72), a member of the HSP70 family.¹⁻⁴ Intracellularly, HSPs perform a variety of chaperoning functions that help to maintain the cytoskeletal integrity and metabolic homeostasis of cells under stress conditions.^{5,6} Members of the HSP70 family, HSP72 and HSP73, respectively,^{7,8} are known to play a role in the intracellular translocation, assembly, and disassembly of other proteins⁹⁻¹³ and have been shown to bind tumor-specific peptides¹⁴ in an adenosine triphosphate-sensitive mode.^{15,16} Recently, evidence has accumulated that HSPs are also localized on the cell surface.¹⁷⁻²¹ The peptide-binding protein that shows high homology to members of the HSP70 family is found on the cell surface in association with major histocompatibility complex (MHC) class-II molecules.¹⁷ Cell surface localization of HSP72, the heat-inducible form of

HSP70, was found on affected retroocular fibroblasts derived from patients suffering from Graves' ophthalmopathy¹⁸ and on virus-infected cells,¹⁹ but not on normal fibroblasts.¹⁸ In BALB/c mice, methylcholanthrene-induced sarcoma cells also express HSP70 on the cell surface.²⁰ Recent data from our group²¹ showed a heat-inducible cell surface expression of HSP72 on different human tumor cell lines. Although HSPs are among the most highly conserved proteins^{22,23} with a wide phylogenetic representation,²⁴ HSPs have been found to act as specific immunogenic determinants expressed on tumor cells.²⁵⁻²⁷ Thus, the question arises as to whether HSPs themselves or HSP-associated peptides act as immunogenic determinants. Recently, immunogenic peptides were found to be associated with HSP70,²⁸ which can be recognized by T cells. In the present report, we addressed the question as to whether interleukin-2 (IL-2)-stimulated natural killer (NK) cells, which are known to play an important role in the elimination of tumors, might also play a role in the recognition of HSPs. The oncolytic function of NK cells can be stimulated by the loss of certain MHC class-I alleles on tumors²⁹⁻³¹ and can be further enhanced by activation with different cytokines, predominantly with IL-2.^{32,33} Here, we show that nonlethal heat shock selectively induces a cell surface expression of an immunogenic HSP72 determinant on human sarcoma cells that persists for at least 96 hours. Furthermore, heat leads to a transient decrease in the MHC class-I expression. Functionally, we show that heat shock leads to an increased susceptibility to lysis by non-MHC-restricted, CD3⁻ NK cells. This phenomenon could be correlated with the heat-inducible cell surface expression of the immunogenic HSP72 determinant²¹ but is not because of a decrease in the MHC class-I expression.

MATERIALS AND METHODS

All cell lines were screened (Mycoplasma tissue culture Genprobe; H. Biermann, Bad Nauheim, Germany) and defined as negative for mycoplasma contaminations.

Peripheral blood lymphocyte (PBL) preparations and generation of Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines

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(B-LCLs). PB obtained from four different healthy human volunteers (A, B, C, and D) was anticoagulated with heparin (Heparin Novo; Novo Nordisk Pharma GmbH, Mainz, Germany). PB mononuclear cells were separated by Ficoll Isopaque (Ficoll Paque; Pharmacia, Uppsala, Sweden) density gradient centrifugation. After separation, PBLs were obtained and incubated in RPMI 1640 (GIBCO, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), 6 mmol/L L-glutamine (GIBCO), and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin; GIBCO). Following the established method of Terasaki and McClelland,³⁴ PBLs derived from healthy human blood donors (A, B, C, and D) were HLA-typed with defined alloantisera and monoclonal HLA-specific antibodies as follows: Donor A: A2, B60(40), B62(15), Bw6, Cw3, DR4, DR13(6), DQ6(1), DQ7(3), DR52/53; Donor B: A2, A29(19), B44(12), B61(40), Bw4, Bw6, Cw2, Cw4, DR7, DQ1, DQw7(3); Donor C: A3, A11, B35, B39(16), Bw6, Cw4, DR1, DR3, DR52, DQ1, DQ2; and Donor D: A24(9), A30(19), B7, B27, Bw4, Bw6, Cw2, Cw7.

A human EBV-transformed B-LCL was established from freshly isolated PBLs derived from donor D. Briefly, PBLs (10×10^6) were incubated in 5 mL RPMI 1640 medium containing 25% FCS, 6 mmol/L L-glutamine, antibiotics (for concentrations, see above), and an equal volume of EBV-containing supernatant (from the EBV producer cell line B95-8; American Type Culture Collection [ATCC], Rockville, MD) for 10 to 14 days. Phytohemagglutinin (PHA-M; Difco, Hamburg, Germany) was added to a final concentration of 1%. After 3 weeks, a permanently growing EBV-transformed B-LCL was generated.

Cell culture of normal and malignant cells. Allogeneic EBV-transformed B-LCLs, K562 cells (a myeloid tumor cell line derived from a patient with chronic myelogenous leukemia in blast phase; ATCC, CCL 243),³⁵ the osteosarcoma cell lines HOS58 (kindly provided by Dr Schmidt, GSF-Institute for Molecular Virology) and MG63 (ATCC, CRL 1427), and the mammary carcinoma cell line MX-1 (kindly provided by Dr Wagner, University of Lübeck and DKFZ Heidelberg, Institute for Experimental Pathology) were grown in RPMI 1640, supplemented with 10% FCS, 6 mmol/L L-glutamine, penicillin, and streptomycin (for concentrations, see above).

Exponentially growing 5838 Ewing's sarcoma (ES) cells, derived from a patient suffering from ES,³⁶ kindly provided by Dr M.L. Meltz (University of Texas, San Antonio, TX), were maintained in RPMI 1640, supplemented with 15% FCS, 6 mmol/L L-glutamine, 0.01 mol/L NaOH (pH 8.5 is essential for growth of tumor cells), and antibiotics (60 IU/mL penicillin and 60 µg/mL streptomycin). As growth parameters, plating efficiency (PE) and multiplicity (N) of these cells under constant culture conditions at 37°C were defined as PE equals 40% to 60% and N equals 2.0 to 2.2. HLA typing of ES cells following the Terasaki method was not possible. Therefore, ES cells were typed by flow cytometry as described below in the Heat Treatment section.

Clonogenic cell surviving assay. Exponentially growing monolayer cells were treated at different temperatures (41.8°C, 42°C, 43°C, 44°C, and 45°C) for varying incubation periods ranging from 0 to 450 minutes in a temperature-controlled waterbath (Haake E3, Karlsruhe, Germany). After treatment, the cells were trypsinized, counted, and plated at dilutions of known cell numbers (10^2 to 10^5 cells/flask) into four replicate flasks. After about 12 days, the colony number was determined, and the cell viability was calculated with corrections for the PE. In addition, cell viability was also tested by using dye exclusion assays (trypan blue, propidium iodide). The temperature of 41.8°C was defined as nonlethal for human tumor and normal cells because, at this temperature, greater than 97% of the cells remained viable in the clonogenic surviving assay³¹ and more than 98% of the cells excluded trypan blue and propidium iodide.

Heat treatment. Exponentially growing cells were treated with

the nonlethal temperature (41.8°C) for 200 minutes in a temperature-controlled waterbath (Haake E3) and were then incubated at 37°C for different time intervals ranging from 0 to 96 hours. The heat dose parameter of 41.8°C was chosen in accordance with data obtained from clonogenic cell surviving assays.²¹

Monoclonal antibodies (MoAbs), indirect immunofluorescence, and FACSscan analysis. Phenotypic characterization of viable effector and target cells was performed by indirect immunofluorescence followed by flowcytometric analysis on a FACSscan instrument (Becton Dickinson and Co, registered trademark for a fluorescence-activated cell sorter, Heidelberg, Germany). Viable cells (1×10^6) were incubated with the following antibodies (final concentration, 5 µg/ 1×10^6) each containing 0.1% sodium azide (NaN₃) at 4°C for 1 hour. The isotype and the specificity of the antibodies is given in parentheses: anti-HSP27 (IgG1), an isotype matched control MoAb for HSP72 (Dianova, Hamburg, Germany); anti-HSP72 (IgG1, RPN1197; Amersham, Braunschweig, Germany); mouse IgG2a isotype-matched control antibody for W6/32 (Dianova), W6/32 (IgG2a; anti-MHC class I); L243 (IgG2a; anti-HLA DR region); OKT3 (IgG2a, anti-CD3) MoAb kindly provided by Dr J. Johnson (München); anti-CD4 (IgG1; Dianova); anti-CD8 (IgG1; Dianova); anti-CD16 (IgG2a; Dianova); anti-CD19 (IgG1; Dianova); anti-CD56 (IgG1; Dianova); anti-CD57 (IgM; Dianova); WT31, an anti- α/β TCR (IgG1; Becton Dickinson); and δ -TCS1, an anti- γ/δ TCR (IgG1; T Cell Sciences, Cambridge, MA). For HLA typing of ES cells, monoclonal and polyclonal antibodies and antisera directed against the following epitopes were used: HLA-A2, A3, A9, A10, A25, A29, A30, A31, A32, and the entire A-region; HLA-B7, B8, B13, B14, B15, B17, B21, B22, B23, B27, B37, B44, Bw4, Bw6; HLA-Cw2, Cw3, Cw4, Cw7; HLA-DR1, DR3, DR5, DR6, DR7, DR8, DR12, DR13, DRw52; and HLA-DQ1, DQ2, DQ3, DQ5, DQ6, DQ7, DQ8, DQ9. After washing twice in phosphate-buffered saline/10% FCS, the cells were stained with a second fluorescein isothiocyanate-conjugated rabbit antimouse Ig antibody (DAKO, Hamburg, Germany) for 1 hour at 4°C. Quantitative flow cytometry was performed on a FACSscan instrument. The percentage of positively stained cells was defined as the difference of the number of specifically stained cells minus the number of cells stained with the isotype-matched control MoAbs (see above). The data obtained from FACSscan analysis represent the mean values of four independent experiments.

Generation of cytotoxic effector cells. Cytotoxic effector cells were generated by incubation of PBLs (50×10^6) from HLA-disparate donors (A, B, and C) with irradiated (60 Gy), heat-treated ES cells (50×10^6) in the presence of recombinant IL-2 (100 IU/mL; EuroCetus, Frankfurt, Germany) for 7 days. For heat treatment of tumor cells, viable ES monolayer cells were heat-treated at nonlethal temperatures (41.8°C for 200 minutes) in a temperature-controlled waterbath.

Separation of effector cell subpopulations. Briefly, PB mononuclear cells were separated into a CD3⁺ T-cell (α/β and γ/δ T-cell receptor [TCR]-positive T cells) and a CD3⁻ (CD56⁺, CD57⁺ NK cells) effector cell population by adherence selection,³⁷ followed by a magnetic bead separation method (Dynabeads M-450; Dynal, Hamburg, Germany) on day 7 after stimulation and expansion with recombinant IL-2 (100 IU/mL). For this purpose, both cell fractions (50 to 100×10^6) generated from PBLs of donor A or B were incubated with a CD3-specific MoAb (OKT3) at 4°C for 1 hour. After being washed twice with phosphate-buffered saline/5% FCS, the cells were incubated with 3 magnetic beads per cell (Dynabeads M-450), which were prewashed twice in RPMI 1640/5% FCS medium and then suspended in 0.5 mL RPMI 1640 medium. After 1 hour of incubation at 4°C, the cells were washed 3 times with 2 mL RPMI 1640 medium and then separated into two cell fractions by magnetic bead separation using the magnetic particle concentrator Dynal-MPC1 (Dynal). CD3⁺ T cells were bound to the magnetic

beads, whereas CD3⁺ cells remained in the supernatant. The magnetic beads were removed from the positively enriched cell population by dissociation using Detach-A-Bead solution (Dyna). After 4 washing steps and an overnight recovery period in RPMI 1640/15% FCS at 37°C, both cell populations were used separately in a cell-mediated lympholysis assay (CML). The phenotype characterization of both cell fractions was performed by FACScan analysis using MoAbs directed against CD19 (IgG1), CD3 (IgG2a), γ/δ TCR (IgG1), CD16 (IgG2a), CD56 (IgG1), and CD57 (IgM). The percentage of γ/δ TCR⁺ T cells was less than 5%; no CD19⁺ B cells were found. Further phenotypic characterization is given in the figure legends.

Cytotoxicity assay. The specificity of IL-2-stimulated effector cells was monitored in a standard ⁵¹Cr release assay.³⁸ The following cells were used as ⁵¹Cr-labeled (5 mCi/mL stock; 0.4 mCi in 0.2 mL RPMI 1640/1 \times 10⁶ cells; NEN-DuPont, Bad Homburg, Germany) target cells (3,000/well), either untreated (37°C) or heat-treated at nonlethal temperatures (41.8°C for 200 minutes, followed by a 4-hour or 12-hour recovery period at 37°C): ES cells, K562 cells, EBV-transformed B-LCLs, and MX-1 cells. After a 2-hour labeling period, the target cells were washed 3 times and adjusted to 10⁴ cells/mL in RPMI 1640/15% FCS medium (CML medium). The cells were coincubated at 37°C for 4 hours in 96-well round-bottomed microtiter plates (Greiner, Nürtingen, Germany) in a total volume of 0.2 mL/well, with cytotoxic effector cells at varying effector-to-target (E/T) ratios. The percentage of specific lysis was calculated as: [(experimental release - spontaneous release) : (maximum release - spontaneous release)] \times 100. Spontaneous release (SR) was assessed by incubating target cells in the absence of effector cells. SR in all experiments was below 20%.

Antibody-blocking assay. Inhibition of lysis by MoAbs directed against antigens expressed on either target (anti-MHC class I, W6/32^{39,40}; anti-HSP72, RPN1197) or effector cells (anti- α/β TCR, WT31)⁴¹ was performed by preincubation of the cells in 0.1-mL aliquots for 2 hours at room temperature. As a control, the isotype-matched MoAb HSP27 was used. The inhibition assays were performed at a final concentration of 10 μ g/1 \times 10⁶ for each antibody. After two washing steps of the antibody-treated cells in CML medium, the cytotoxicity assay was performed as described above.

RESULTS

Heat treatment of sarcoma cells leads to HSP72 cell surface expression. A clonogenic cell surviving assay was performed to define nonlethal heat dose parameters for adherent cells. At a temperature of 41.8°C, the cell viability as well as the capacity for clonogenic cell growth of all tested tumor cell lines (ES, HOS58, MG63, and MX-1) and of normal fibroblasts was more than 98% even after heating periods greater than 450 minutes. In contrast, heat exposure of cells at temperatures above 42°C reduced the clonogenic cell viability of neoplastic and normal cells.²¹ The viability of non-adherent cells (PBLs, B-LCLs, and PHA blasts) at 41.8°C (200 minutes) was more than 97%, as determined by trypan blue exclusion. For all further investigations, the nonlethal temperature of 41.8°C was used. Although total protein synthesis is reduced after heat shock, synthesis of HSP72, the major heat-inducible form of HSP70, is strongly induced. Indirect immunofluorescence studies were performed to compare cell surface expression of different molecules on either untreated or heat-treated cells. The results of a comparative study on cell surface expression of HSP72 and MHC class-I molecules on neoplastic human cell types (ES, HOS58, MG63 sarcoma cells, and MX-1 carcinoma cells)

and on cells derived from healthy human individuals (PBLs, fibroblasts, PHA blasts, and B-LCLs) before and after nonlethal heat treatment are summarized in Table 1. Untreated tumor and normal cells show no significant HSP72 cell surface staining. After single nonlethal heat shock (41.8°C for 200 min) and a recovery period at 37°C (4 hours) up to one third of viable sarcoma cells was positively stained using HSP72 MoAb. In contrast, MX-1 carcinoma cells and cells derived from healthy human individuals (PBLs, fibroblasts, PHA blasts, and B-LCLs) showed no HSP72 cell surface expression under these heating conditions. Recently, we showed that coincubation of untreated sarcoma cells with HSP72 containing supernatants of lethally heat-shocked cells does not result in an unspecific HSP72 cell surface expression.²¹ Therefore, adventitious deposition of HSP72 released by dead cells that nonspecifically binds onto live cells is very unlikely. A transient downregulation in the percentage and the fluorescence intensity of MHC class-I molecules after heat shock was observed only with tumor cells, not with normal cells. Kinetic data of flowcytometric analysis indicate that the sarcoma-specific HSP72 cell surface expression peaks 4 hours after nonlethal heat shock and persists for at least 96 hours (Table 2). A transient reduction in the MHC class-I expression selectively on sarcoma cells is observed between 3 and 6 hours after nonlethal heat shock. After a 12-hour recovery period at 37°C, the MHC class-I expression reaches its initial levels. Similar data were obtained with other sarcoma cells such as HOS58 and MG63 (data not shown). With respect to these findings, the functional assays were performed after nonlethal heat shock, followed by a 4-hour and 12-hour recovery period at 37°C.

Heat-induced increased susceptibility to lysis of sarcoma cells is mediated by HLA-disparate effector cells. The ES tumor cells were HLA-typed by flow cytometry as A25(10), A29(19), and DQ7(3). No HLA-DR (using L243 MoAb), HLA-B (using MoAbs directed against Bw4 or Bw6), or HLA-C (using 4 different MoAbs directed against HLA-C alleles) reactivity was found. Cytotoxic activity of HLA-disparate effector cells was tested in a CML assay against either untreated (37°C) or heat-treated (41.8°C for 200 minutes and 37°C for 4 hours) ES cells as tumor target cells. Donors A, B, and C were chosen to generate cytotoxic effector cells because they share one [DQ7(3) is shared between effector cells A and ES target cells], two [DQ7(3) and A29(19) is shared between effector cells B and ES target cells], or no [no HLA allele is shared between effector cells C and ES target cells] HLA alleles with the ES target cells. Figure 1 shows for all three HLA-disparate effector cell populations (A, B, and C) comparable lysis patterns. The lysis against nonlethally heat-treated (41.8°C for 200 minutes and 37°C for 4 hours) ES cells compared with that for untreated (37°C) ES cells was increased more than twofold. In three independent experiments using identical experimental parameters, lysis of heat-treated ES cells was always significantly stronger compared with that of untreated ES cells.

Lysis of heat-treated sarcoma cells is mediated by CD3⁺ NK cells. With respect to the increased susceptibility of heat-shocked sarcoma cells by HLA-disparate effector cells, we investigated the question of whether this cytotoxic response is MHC-independent. Using the immunomagnetic

Table 1. Comparative Flow Cytometric Analysis of HSP27 (isotype-matched Negative Control Antibody for HSP72, IgG1), HSP72 (IgG1), and MHC Class-I (W6/32 MoAb, IgG2a) Cell Surface Expression on Neoplastic Versus Normal Cells Either Untreated (37°C) or Heat-Shocked at Nonlethal Temperatures

	MoAbs					
	HSP27		HSP72		W6/32	
	37°C	41.8°C*	37°C	41.8°C*	37°C	41.8°C*
Neoplastic cells						
ES	1.5 ± 0.7 (24)	1.2 ± 0.8 (26)	3.2 ± 0.8 (31)	31.4 ± 1.9 (117)†	83.7 ± 3.5 (123)	70.5 ± 2.8 (94)†
HOS58	1.8 ± 0.3 (19)	2.1 ± 0.5 (19)	2.3 ± 0.4 (26)	25.9 ± 3.9 (90)†	73.2 ± 2.9 (183)	54.1 ± 3.2 (143)†
MG63	1.6 ± 0.6 (11)	1.9 ± 0.4 (11)	1.9 ± 0.6 (15)	15.7 ± 3.0 (52)†	90.0 ± 4.1 (201)	78.4 ± 3.3 (172)†
MX-1	2.3 ± 0.5 (10)	2.2 ± 0.5 (12)	2.8 ± 0.4 (12)	2.3 ± 0.6 (12)	99.8 ± 2.4 (126)	99.2 ± 2.7 (87)
Normal cells						
PBL	0.3 ± 0.1 (11)	0.2 ± 0.1 (10)	0.0 ± 0.0 (10)	0.0 ± 0.0 (14)	92.7 ± 3.2 (231)	90.8 ± 4.0 (238)
Fibro	1.1 ± 0.3 (11)	1.0 ± 0.2 (12)	1.2 ± 0.1 (12)	2.2 ± 0.2 (11)	96.3 ± 3.7 (203)	97.4 ± 2.9 (251)
PHA	0.3 ± 0.1 (10)	0.2 ± 0.1 (10)	0.3 ± 0.1 (9)	0.3 ± 0.1 (10)	94.8 ± 2.9 (272)	95.5 ± 3.2 (259)
B-LCL	0.3 ± 0.2 (12)	0.4 ± 0.1 (13)	0.3 ± 0.1 (16)	0.3 ± 0.1 (13)	97.9 ± 4.3 (294)	96.0 ± 2.9 (307)

Values shown are the percentage of positively stained cells ± standard deviation (SD) with the average mean fluorescence intensity in parentheses. A mouse Ig2a isotype-matched control antibody was used for MHC class-I expression (data not shown). Neoplastic cells included ES cells (ES), osteosarcoma cells (HOS58, MG63), and Mamma carcinoma cells (MX-1). Normal cells included PBLs, fibroblasts (Fibro), phytohemagglutinin stimulated PBLs (PHA), EBV-transformed B-cells (B-LCLs) derived from healthy human individuals.

* Treatment was at 41.8°C for 200 minutes and at 37°C for 4 hours (as described in Materials and Methods).

† Significantly different from control levels ($P < .01$) with Student's *t*-test.

bead separation method after adherence selection, a stimulated mixed effector cell population was divided into a CD3⁺ T-cell population and a CD3⁻ NK cell population. Purity of both cell populations was more than 95% as shown by FAC-Scan analysis using CD3 and WT31 MoAbs as T-cell markers and CD16, CD56, and CD57 MoAbs as NK cell markers (Fig 2, legend). The percentage of γ/δ TCR⁺ T cells was less than 5% in both cell fractions. The results derived from

CML assays using unseparated (Fig 2A) and separated CD3⁺ (Fig 2B) and CD3⁻ (Fig 2C) effector cells against either untreated (37°C) or nonlethally heat-treated (heat shock [hs], 41.8°C for 200 minutes and 37°C for 4 hours) ES and K562 cells are shown in Fig 2. Here, we show that lysis of untreated and heat-treated K562 cells is mediated predominantly by the CD3⁻ NK effector cells but not by CD3⁺ T

Table 2. Flow Cytometric Analysis of HSP72 and MHC Class-I (W6/32 MoAb) Cell Surface Expression on ES Cells After a Single Nonlethal Heat Treatment Followed by Different Incubation Periods (0 to 96 Hours) at 37°C

Recovery Periods After 41.8°C* (no. of hours at 37°C)	MoAbs	
	HSP72	W6/32
0	4.2 ± 1.3 (62)	84.2 ± 2.6 (110)
1	15.7 ± 1.8 (98)†	82.4 ± 2.8 (103)
2	20.9 ± 1.7 (119)†	83.7 ± 2.4 (103)
3	28.3 ± 2.1 (120)†	73.3 ± 2.9 (91)†
4	31.4 ± 1.9 (117)†	70.5 ± 2.8 (94)†
5	26.4 ± 1.8 (104)†	68.9 ± 3.2 (98)†
6	27.4 ± 2.7 (109)†	72.9 ± 2.3 (98)†
8	27.6 ± 1.4 (100)†	84.0 ± 2.2 (118)
12	27.3 ± 1.5 (92)†	83.9 ± 2.4 (117)
24	26.6 ± 1.8 (89)†	83.7 ± 2.7 (110)
48	19.3 ± 1.3 (86)†	84.1 ± 2.2 (121)
72	19.7 ± 1.9 (90)†	83.4 ± 2.6 (119)
96	17.3 ± 2.1 (87)†	84.8 ± 2.9 (122)

Values shown are the percentage of positively stained ES cells ± standard deviation (SD) with the mean fluorescence intensity in parentheses. Anti-HSP27 (IgG1) and mouse IgG2a MoAbs were used as isotype-matched control antibodies for HSP72 and W6/32 MoAbs.

* Treatment was at 41.8°C for 200 minutes.

† Significantly different from control levels ($P < .01$) with Student's *t*-test.

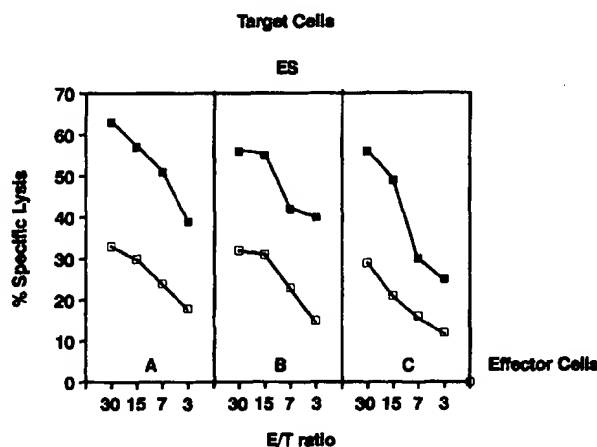


Fig 1. Increased sensitivity of nonlethally heat-shocked sarcoma cells to lysis by HLA-disparate effector cell populations. Cytotoxic activity of HLA-disparate effector cells (donors A, B, and C) showed no obvious differences in the lysis pattern. ⁵¹Cr-labeled, heat-treated ES cells (■) were lysed to a much higher degree (about twofold), as compared with untreated ES target cells (□), by all three effector populations. This effect was titratable at varying E/T ratios ranging from 30:1 to 3:1. Each data point represents the mean value of three independent experiments; ± SD was always less than 10%. SR of untreated and heat-treated target cells was identical and below 20%. Similar lysis patterns were obtained with other sarcoma cell lines (HOS58, MG63) that were either untreated or nonlethally heat-treated (data not shown).

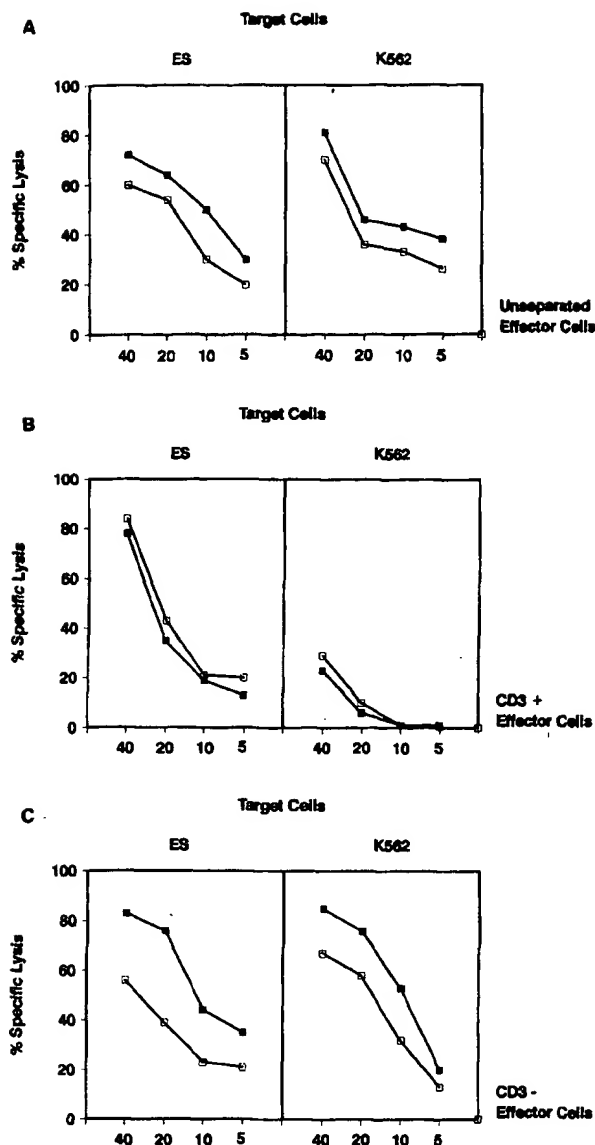


Fig 2. Increased sensitivity to lysis of heat-shocked ES and K562 cells is mediated by non-MHC-restricted, CD3⁺ NK cells, but not by CD3⁺ CTLs. Cytotoxic activity of (A) a mixed effector cell population (83%, CD3; 22%, CD16; 18%, CD56; 16%, CD57; 5%, γ/δ), (B) a CD3⁺ CTL-enriched (97%, CD3; 7%, CD16; 9%, CD56; 15%, CD57; 4%, γ/δ TCR), and (C) a CD3⁺ NK-enriched (5%, CD3; 43%, CD16; 45%, CD56; 32%, CD57; 1%, γ/δ TCR) effector cell population was tested separately to untreated (□) and heat-treated (■) K562 and ES target cells. Lysis of K562 cells, either untreated or heat-treated, was only observed with the CD3⁺ NK-enriched effector cell population, whereas the CTL-mediated lysis of K562 target cells was weak. The enhanced lysis of heat-treated ES cells was also mediated by the NK-enriched population. Untreated ES cells were lysed by both effector cell populations. E/T ratios ranged from 40:1 to 5:1. Each data point represents the mean value of three independent experiments; \pm SD was always less than 8%. SR of untreated and heat-treated target cells was comparable and was always below 20%.

lymphocytes. The enhanced lysis of heat-shocked K562 cells is expressed more clearly by the NK population. The lysis of untreated ES cells is mediated by CD3⁺ T lymphocytes as well as by CD3⁺ NK cells. However, the enhanced sensi-

tivity of heat-shocked ES cells is mediated predominantly by CD3⁺ NK cells.

Anti-HSP72 MoAb inhibits increased lysis of heat-treated K562 and ES cells. Antibody-blocking CML experiments using an NK-enriched effector cell population (phenotypic characterization, see the legend for Fig 3) were performed to define a possible recognition structure that is expressed on the cell surface of heat-shocked neoplastic cells. To exclude the effect of a transient MHC class-I downregulation on tumor target cells (4 hours after nonlethal heat shock), the lysis of untreated and heat-treated (41.8°C for 200 minutes) cells was compared after a 12-hour recovery period at 37°C (Table 2) when the MHC class-I expression has reached its initial level. Similar to the data presented in Figs 1 and 2 (important to note the 4-hour recovery period at 37°C), the lysis of heat-shocked ES (Fig 3A) and K562 (Fig 3B) cells followed by a 12-hour recovery period again was significantly higher as compared with the lysis of untreated (37°C) cells (Fig 3). Most important, this elevated lysis of heat-shocked neoplastic cells could be inhibited by preincubation of the target cells using HSP72-specific MoAb, whereas an isotype-matched control antibody directed against anti-HSP27 has no inhibitory effect. Further evidence that HSP72 is a relevant recognition structure for NK effector cells is given by the results obtained with the control tumor cell line MX-1 (Fig 3C). These cells do not express HSP72 molecules on their cell surface (Table 1), and their lysis was not enhanced after heat shock. Neither the HSP72 MoAb nor the isotype-matched control antibody (HSP27) had an inhibitory effect on the lysis of these cells after heat shock. Cytotoxic T lymphocyte (CTL) recognition of MX-1 tumor cells could be excluded, because blocking of MHC-restricted recognition by anti-MHC class-I MoAb or by anti-TCR MoAb has also no inhibitory effect. The anti-MHC class-I MoAb (W6/32) that is known to mask MHC class-I gene products^{42,43} does not influence the lysis of heat-treated ES and K562 cells. A role of L243 MoAb (directed against HLA-DR region) that could mask MHC class-II gene products could be excluded, because no HLA-DR expression was detectable on the surface of ES and K562 cells. Preincubation of the effector cell population using WT31 MoAb (directed against α/β TCR⁺ T lymphocytes),⁴¹ has no inhibitory effect on the lysis of heat-shocked neoplastic cells. These findings support the suggestion that the elevated lysis of heat-treated ES and of K562 cells is not mediated by MHC class-I-restricted T lymphocytes. As a control for CTL activity in the NK-enriched effector cells, we show that the lysis of allogeneic B-LCLs (Fig 3D) is inhibited by incubation of the effector cells using an anti-TCR-specific MoAb (WT31) or by blocking of MHC class-I molecules on the target cells using W6/32 MoAb.

DISCUSSION

In the present study, we provide direct evidence that a heat-inducible HSP72 cell surface expression is correlated with increased sensitivity of tumor cells against non-MHC-restricted NK cells. Recently, MHC-independent γ/δ TCR⁺, CD3⁺ T cells, which are considered to play a role in the defense against tumor cells,⁴⁴ are shown to recognize cell surface-expressed HSPs of the HSP60 and HSP70 fami-

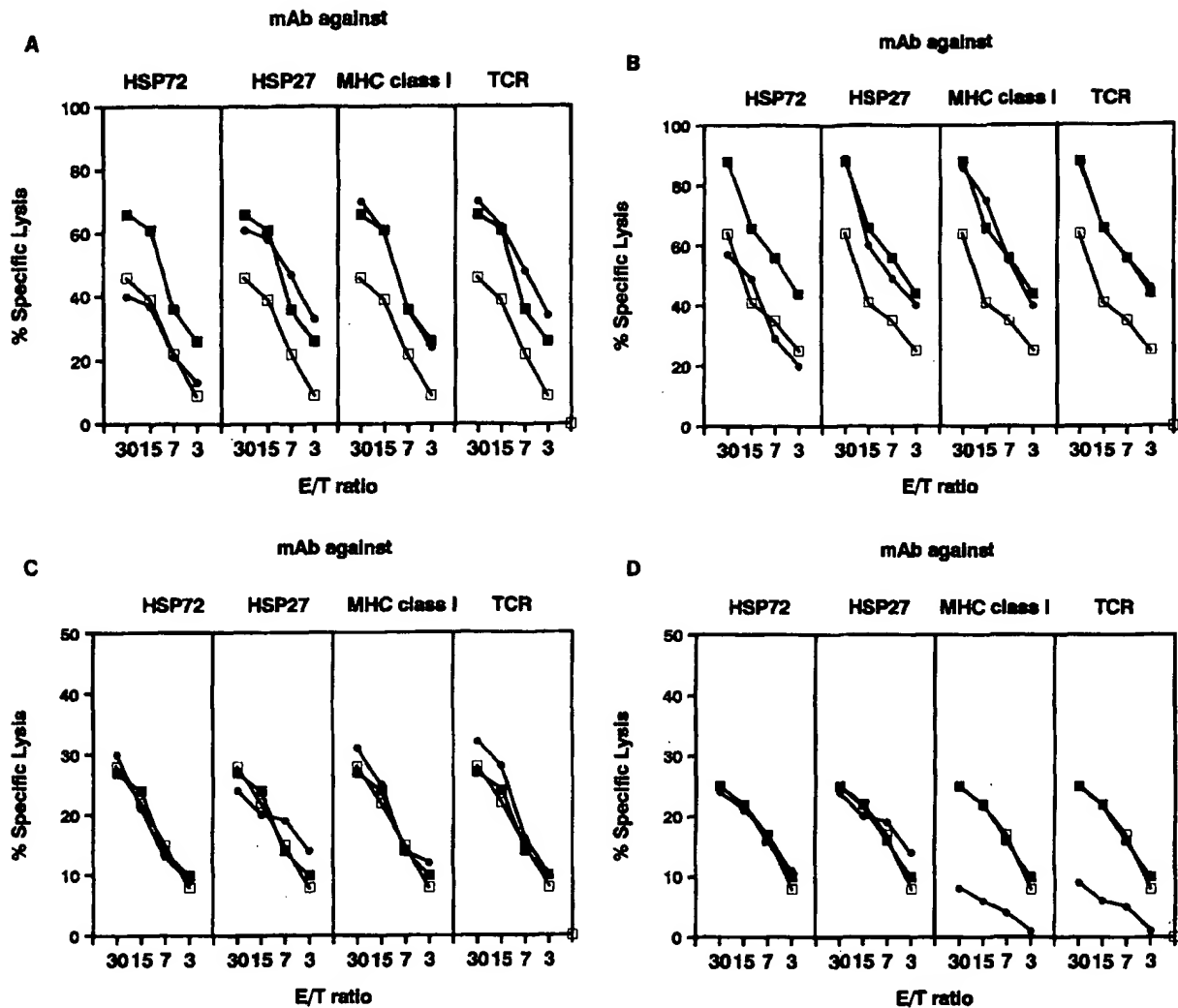


Fig 3. An HSP72-associated determinant acts as a recognition structure for NK-enriched (24%, CD3; 57%, CD16; 54%, CD56; 29%, CD57; 2%, γ/δ) effector cells on heat-shocked sarcoma cells. Effect of HSP72-specific (IgG1), HSP27-specific (IgG1, isotype-matched control), and MHC class-I-specific (W6/32; IgG2a) MoAbs on the lysis of heat-treated target cells, ie, ES (A), K562 (B), MX-1 (C), and allogeneic B-LCLs (D). (\square), lysis of untreated (37°C) target cells; (\blacksquare) lysis of nonlethally heat-shocked (41.8°C for 200 minutes and 37°C for 12 hours) target cells; (\bullet) lysis of heat-shocked target cells after antibody blocking. E/T ratios ranged from 30:1 to 3:1. Each data point represents the mean value of two independent experiments; \pm SD was always less than 8%. SR of untreated and heat-treated target cells was identical and was below 20%. Lysis of ES and K562 tumor cells that express HSP72 (A and B) is inhibited using HSP72 MoAb; lysis of MX-1 carcinoma cells (C) and B-LCLs (D) that do not express HSP72 is not inhibited using HSP72 MoAb. The isotype-matched control antibody HSP27 (IgG1) has no inhibitory effect. Inhibition of the CTL-mediated lysis by MHC class-I-specific MoAb W6/32 (IgG2a) or by α/β TCR-specific MoAb WT31 (IgG1) has no inhibitory effect on the lysis of heat-shocked ES, K562, and MX-1 tumor cells (A, B, and C), whereas the lysis of heat-shocked allogeneic B-LCLs was inhibited by W6/32 and by WT31. Elevated lysis of other nonlethally heat-shocked sarcoma cells (HOS58, MG63 osteosarcoma cells) was also inhibited by HSP72 MoAb, but not by MHC class-I-specific (W6/32) or α/β TCR-specific (WT31) MoAbs (data not shown).

lies.^{45,46} For the recognition of the heat-inducible HSP72 epitope on sarcoma cells, γ/δ TCR⁺ T lymphocytes can be excluded, because the amount of γ/δ T lymphocytes within the stimulated effector cell population (as determined by FACScan analysis) was always below 5%. Cytotoxic activity mediated by CD3⁺ lymphokine-activated killer cells^{47,48} is also not very likely because, after separation of the effector cell population in a CD3⁺ and a CD3⁻ cell population, reactivity against heat-shocked tumor target cells was only found within the CD3⁻ cell fraction. The CD3⁻ cell population that was shown to be responsible for the increased lysis of heat-

treated sarcoma cells and of classical NK target cells (K562) expresses NK markers CD16, CD56, and CD57. Lysis of untreated sarcoma cells is mediated by CD3⁻ and CD3⁺ effector cells. The transient reduction in the MHC class-I expression on tumor cells after heat shock is a possible explanation for the decreased lysis of heat-treated tumor cells by CD3⁺ T lymphocytes, whereas there is no influence on the elevated lysis of heat-treated sarcoma cells mediated by CD3⁻ NK effector cells. Preincubation of tumor as well as allogeneic target cells with MHC class-I-specific MoAb (W6/32), which is known to block the CTL-mediated cyto-

toxicity,^{39,40} inhibits the lysis of allogeneic target cells but not the lysis of heat-shocked sarcoma and K562 cells. These findings are supported by blocking of α/β TCR⁺ T cells using WT31 MoAb.⁴¹ Again, only the lysis of allogeneic target cells, but not that of heat-shocked tumor and NK target cells, was blocked by this TCR-specific antibody. In contrast to the majority of cytotoxic responses of CTLs, which are known to be MHC-restricted, it has been shown that low levels of MHC class-I expression on tumor cells are associated with a higher susceptibility of lysis by NK cells.^{42,49-51} Our observations that heat shock leads to a transient decrease in the MHC class-I expression could not be correlated with the elevated lysis of heat-shocked tumor cells that express HSP72. Therefore, we speculate that MHC-dependent⁴³ and MHC-independent^{52,53} mechanisms of NK recognition have to exist. Despite the fact that certain HLA class-I alleles could be identified that act as negative signals for NK recognition on normal cells,⁴³ it is still not clear which target structures on tumor cells can act as positive recognition signals for NK cells. Our results derived from antibody-blocking studies using an HSP72-specific MoAb, suggest that a heat-inducible HSP72 epitope on sarcoma cells might act as a positive recognition structure for NK cells. This hypothesis is strongly supported by data derived from CML assays using heat-shocked carcinoma cells (MX-1) that showed no HSP72 cell surface expression after heat shock. The NK-mediated lysis of these target cells was not increased after heat shock. Therefore, we conclude that the increased sensitivity of heat-shocked sarcoma cells is caused by the HSP72 cell surface expression and is not a nonspecific effect of heat treatment. Furthermore, the increased lysis of heat-treated K562 cells and, to a lower extent, the lysis of untreated K562 cells were inhibited by HSP72 MoAb. These data led us to the hypothesis that heat-treated sarcoma cells and K562 cells have to share at least one recognition structure for NK cells, which seems to be closely associated with HSP72. Results derived from cold target inhibition studies support this hypothesis (data not shown). Recently, we showed that a 72-kD protein could be immunoprecipitated from the cellular membrane of different heat-treated tumor cells but not of normal cells by using the HSP72 MoAb.²¹ These data are in line with observations of other groups that HSP72 cell surface localization was only found on affected cell types such as tumor cells,⁵⁴ human immunodeficiency virus-¹⁹ and human T-cell lymphotropic virus-infected cells,⁵⁵ mycobacterial-infected cells,⁵⁶ and affected retroocular fibroblasts obtained from patients suffering from Graves' ophthalmopathy.¹⁸ In this context, it is important to note that HSPs are discussed to act as immunogenic determinants for different effector cell mechanisms.⁵⁷ Ullrich et al⁵⁸ and Srivastava et al⁵⁹ raised the hypothesis that HSPs homologous to members of the HSP90 and HSP70 family are recognized as tumor antigens in mice by CTLs. Carbohydrate side chains as antigenic determinants on these HSPs can be excluded, because none of them is glycosylated.⁶⁰ Tamura et al⁶¹ showed that improved immunogenicity of Ha-ras-transfected fetal rat fibroblasts mediated by CD4 and CD8 double-negative T cells could be correlated with the presence of an HSP70 cognate protein on the cell surface of tumor cells.

In conclusion, our data show that nonlethal heat shock

induces a cell surface expression of HSP72 that persists for at least 96 hours. This immunogenic epitope is selectively expressed on human sarcoma cells and can be correlated with NK recognition. Interestingly, normal cells and the MX-1 mammary carcinoma cell line fail to express this recognition structure. Additionally, heat treatment leads to a transient reduction in the MHC class-I expression. The HSP72 cell surface expression selectively on tumor cells could act as one possible recognition structure for an NK subpopulation. Our in vitro data that NK cells are relevant for recognition of heat-treated tumor cells are in line with recently published observations of an in vivo study that tumors are spontaneously infiltrated and killed by NK cells.⁶² Furthermore, inhibition of metastasis has also been shown to correlate with the activity of NK cells.^{63,64} NK-depleted mice seem to be more susceptible for rapid tumor growth after tumor transplantation.⁶⁵ Therefore, besides MHC-restricted effector mechanisms, such as CTL activities, non-MHC-restricted NK cells must also be considered as possible mediators for an antitumor immune response.

REFERENCES

1. Lindquist S: The heat-shock response. *Annu Rev Biochem* 55:1151, 1986
2. Welch WJ, Suhan JP: Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. *J Cell Biol* 103:2035, 1986
3. Craig EA: The heat shock response. *Crit Rev Biochem* 18:239, 1985
4. Ashburner M, Bonner JJ: The induction of gene activity in drosophila by heat shock. *Cell* 17:241, 1979
5. Ellis RJ, Hemmingsen SM: Molecular chaperones: Proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem Sci* 14:339, 1989
6. Welch WJ: Mammalian stress response: Cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol Rev* 72:1063, 1992
7. Welch WJ, Feramisco JR: Purification of the major mammalian heat shock proteins. *J Biol Chem* 257:14949, 1982
8. Welch WJ, Feramisco JR: Nuclear and nucleolar localization of the 72,000-dalton heat shock protein in heat-shocked mammalian cells. *J Biol Chem* 259:4501, 1984
9. Pelham HR: Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell* 46:959, 1986
10. Chirico WJ, Waters MG, Blobel G: 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature* 332:805, 1988
11. Deshaies RJ, Koch BD, Werner-Washburne M, Craig EA, Schekman R: A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* 332:800, 1988
12. Zimmermann R, Sagstetter M, Lewis MJ, Pelham HR: Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. *EMBO J* 7:2875, 1988
13. Hightower LE: Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides. *J Cell Physiol* 102:407, 1980
14. Srivastava PK, Maki RG: Stress-induced proteins in immune response to cancer. *Curr Top Microbiol Immunol* 167:109, 1991
15. Flynn G-C, Chappell T-G, Rothman J-E: Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science* 245:385, 1989

16. Flynn G-C, Pohl J, Flocco M-T, Rothman J-E: Peptide-binding specificity of the molecular chaperone BiP. *Nature* 353:726, 1991
17. Vanbuskirk A, Crump BL, Margoliash E, Pierce SK: A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. *J Exp Med* 170:1799, 1989
18. Heufelder AE, Wenzel BE, Bahn RS: Cell surface localization of a 72 kilodalton heat shock protein in retroocular fibroblasts from patients with Graves' ophthalmopathy. *J Clin Endocrinol Metab* 74:732, 1992
19. Di-Cesare S, Poccia F, Mastino A, Colizzi V: Surface expressed heat-shock proteins by stressed or human immunodeficiency virus (HIV)-infected lymphoid cells represent the target for antibody-dependent cellular cytotoxicity. *Immunology* 76:341, 1992
20. Srivastava PK: Peptide-binding heat shock proteins in the endoplasmic reticulum: Role in immune response to cancer and in antigen presentation. *Adv Cancer Res* 62:153, 1993
21. Multhoff G, Botzler C, Wiesnet M, Muller E, Meier T, Wilmanns W, Issels RD: A stress-inducible 72 kDa heat shock protein (HSP72) is expressed on the cell surface of human tumor cells, but not on normal cells. *Int J Cancer* 61:272, 1995
22. Ritossa F-M: A new puffing pattern induced by a temperature shock and DNP in *Drosophila*. *Experientia* 18:571, 1962
23. Schlesinger MJ, Ashburner M, Tissieres A (eds): *Heat Shock: From Bacteria to Man*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1982
24. Bienz M, Pelham HR: Mechanisms of heat-shock gene activation in higher eukaryotes. *Adv Genet* 24:31, 1987
25. Young D, Lathigra R, Hendrix R, Sweetser D, Young RA: Stress proteins are immune targets in leprosy and tuberculosis. *Proc Natl Acad Sci USA* 85:4267, 1988
26. Lamb JR, Bal V, Rothbard JB, Mehlert A, Mendez-Samperio P, Young DB: The mycobacterial GroEL stress protein: A common target of T-cell recognition in infection and autoimmunity. *J Autoimmun* 2:93, 1989
27. Young RA: Stress proteins and immunology. *Annu Rev Immunol* 8:401, 1990
28. Uono H, Srivastava PK: Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* 178:1391, 1993
29. Ciccone E, Pende D, Viale O, Tambussi G, Ferrini S, Biassoni R, Longo A, Moretta A, Moretta L: Specific recognition of human CD3- CD16+ natural killer cells requires the expression of an autosomal recessive gene on target cells. *J Exp Med* 172:47, 1990
30. Raulat DH: A sense of something missing. *Nature* 358:21, 1992
31. Versteeg R, Kruse-Wolters KM, Plomp AC, van Leeuwen A, Stam NJ, Ploegh HL, Ruiter DJ, Schrier PI: Suppression of class I human histocompatibility leukocyte antigen by c-myc is locus specific. *J Exp Med* 170:621, 1989
32. O'Shea J, Ortaldo JR: The natural immune system, in Lewis CE, O'McGee D (eds): *The Natural Killer Cell*. New York, NY, Oxford University, 1992, p 1
33. Vujanovic NL, Rabinovich H, Lee YJ, Herberman RB, Whiteside TL: Distinct phenotypic and functional characteristics of human natural killer cells obtained by rapid interleucine 2-induced adherence to plastic. *Cell Immunol* 151:133, 1993
34. Terasaki PI, McClelland JD: Microdroplet assay of human serum cytotoxicity. *Nature* 204:998, 1964
35. Luzzio BC, Luzzio BB: Human chronic myelogenous leukemia cell-line with positive Philadelphia Chromosome. *Blood* 45:321, 1975
36. McCoy JL, Jerome LJ, Cannon GB, Pomeroy TC, Connor RJ, Oldham RK, Weese JL, Herberman RB: Leukocyte migration inhibition in patients with Ewing's sarcoma by 3-M potassium chloride extracts of fresh and tissue-cultured Ewing's sarcomas. *J Natl Cancer Inst* 59:1119, 1977
37. Multhoff G, Meier T, Botzler C, Wiesnet M, Allenbacher A, Wilmanns W, Issels RD: Differential effects of ifosfamide on the capacity of cytotoxic T lymphocytes and natural killer cells to lyse their target cells correlate with intracellular glutathione levels. *Blood* 85:2124, 1995
38. Macdonald HR, Engers HD, Cerottini JC, Brunner KT: Generation of cytotoxic T lymphocytes in vitro. II. Effect of repeated exposure to alloantigens on the cytotoxic activity of long-term mixed leukocyte cultures. *J Exp Med* 140:718, 1974
39. Parham P, Androlewicz MJ, Brodsky FM, Holmes NJ, Ways JP: Monoclonal antibodies: Purification, fragmentation and application to structural and functional studies of class I MHC antigens. *J Immunol Methods* 53:133, 1982
40. McMichael AJ, Parham P, Brodsky FM, Pilch JR: Influenza virus-specific cytotoxic T lymphocytes recognize HLA-molecules. Blocking by monoclonal anti-HLA antibodies. *J Exp Med* 152:195S, 1980
41. Spits H, Borst J, Tax W, Capel PJ, Terhorst C, de-Vries JE: Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T cell receptor for antigen. *J Immunol* 135:1922, 1985
42. Lobo PI, Spencer CE: Use of anti-HLA antibodies to mask major histocompatibility complex gene products on tumor cells can enhance susceptibility of these cells to lysis by natural killer cells. *J Clin Invest* 83:278, 1989
43. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, Barbaresi M, Ciccone E, Moretta L: P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I protected cells in NK clones displaying different specificities. *J Exp Med* 178:597, 1993
44. Ericsson PO, Hansson J, Widegren B, Dohlsten M, Sjogren HO, Hedlund G: In vivo induction of gamma/delta T cells with highly potent and selective anti-tumor cytotoxicity. *Eur J Immunol* 21:2797, 1991
45. Rajasekar R, Sim GK, Augustin A: Self heat shock and gamma delta T-cell reactivity. *Proc Natl Acad Sci USA* 87:1767, 1990
46. Kaur I, Voss SD, Gupta RS, Schell K, Fisch P, Sondel PM: Human peripheral gamma delta T cells recognize hsp60 molecules on Daudi Burkitt's lymphoma cells. *J Immunol* 150:2046, 1993
47. Schmidt RE, Murray C, Daley JF, Schlossman SF, Ritz J: A subset of natural killer cells in peripheral blood displays a mature T cell phenotype. *J Exp Med* 164:351, 1986
48. Uciechowski P, Werfel T, Leo R, Gessner JE, Schubert J, Schmidt RE: Analysis of CD16+dim and CD16+bright lymphocytes—Comparison of peripheral and clonal non-MHC-restricted T cells and NK cells. *Immunobiology* 185:28, 1992
49. Herberman RB, Ortaldo JR: Natural killer cells: The role in defence against disease. *Science* 214:24, 1981
50. Kärre K, Ljunggren HG, Piontek G, Kiessling R: Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319:675, 1986
51. Storkus WJ, Howell DN, Salter RD, Dawson JR, Cresswell P: NK susceptibility varies inversely with target cell class I HLA antigen expression. *J Immunol* 138:1657, 1987
52. Nishimura M, Mitsunaga S, Akaza T, Mitomi Y, Juji T: Protection against natural killer cells by interferon gamma treatment of K562 cells cannot be explained by augmented major histocompatibility complex class I expression. *Immunology* 83:75, 1994
53. Haridas V, Saxena RK: Changes in LAK susceptibility of tumor cells as their MHC class I antigen expression levels regenerate after treatment at pH 3.0. *Immunol Lett* 44:7, 1995
54. Ferrarini M, Heltai S, Zocchi MR, Rugarli C: Unusual expression and localization of heat-shock proteins in human tumor cells. *Int J Cancer* 51:613, 1992
55. Chouchane L, Bowers FS, Sawadikosol S, Simpson RM, Kindt TJ: Heat shock proteins expressed on the surface of human

T cell leukemia virus type I-infected cell lines induce autoantibodies in rabbits. *J Infect Disease* 169:253, 1994

56. Wand-Würtenberger A, Schoel B, Ivanyi J, Kaufmann SHE: Surface expression by mononuclear phagocytes of an epitope shared with mycobacterial heat shock protein 60. *Eur J Immunol* 21:1089, 1991

57. Fuller KJ, Issels RD, Slosman DO, Guillet J-G, Polla BS: Cancer and the heat shock response. *Eur J Cancer* 30:1884, 1994

58. Ullrich SJ, Robinson EA, Law LW, Willingham M, Appella E: A mouse tumor-specific transplantation antigen is a heat shock-related protein. *Proc Natl Acad Sci USA* 83:3121, 1986

59. Srivastava PK, Deleo AB, Old LJ: Tumor rejection antigens of chemically induced sarcomas of inbred mice. *Proc Natl Acad Sci USA* 83:3407, 1986

60. Li Z, Srivastava PK: Tumor rejection antigen gp96/grp94 is an ATPase: Implications for protein folding and antigen presentation. *EMBO J* 12:3143, 1993

61. Tamura Y, Tsuboi N, Sato N, Kikuchi K: 70 kDa heat shock cognate protein is a possible target for the host's anti-tumor immunity. *J Immunol* 151:5516, 1993

62. Kurosawa S, Matsuzaki G, Harada M, Ando T, Nomoto K: Early appearance and activation of natural killer cells in tumor-infiltrating lymphoid cells during tumor development. *Eur J Immunol* 23:1029, 1993

63. Nishimura T, Ohta S, Sato N, Togashi Y, Goto M, Hashimoto Y: Combination tumor immunotherapy with recombinant tumor necrosis factor and recombinant interleukin-2 in mice. *Int J Cancer* 40:255, 1987

64. Lafreniere R, Rosenberg SA: Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin 2. *Cancer Res* 45:3735, 1985

65. Talmadge JE, Meyers KM, Prieur DJ, Starkey JR: Role of NK cells in tumour growth and metastasis in beige mice. *Nature* 284:622, 1980



DIFFERENTIAL Hsp70 PLASMA-MEMBRANE EXPRESSION ON PRIMARY HUMAN TUMORS AND METASTASES IN MICE WITH SEVERE COMBINED IMMUNODEFICIENCY

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To study the role of cell-surface expression of a tumor-selective heat-shock protein 70 (Hsp70) *in vivo*, the colon-carcinoma cell line CX2, and the clonal sub-lines CX⁺ and CX[−], which differ in Hsp70 cell-surface expression, but not in MHC and adhesion-molecule expression, were implanted into immunodeficient SCID/beige mice by s.c., i.p., i.v. and orthotopic (o.t.) inoculation. On day 18 after s.c. injection, all animals developed s.c. tumors, ranging in size from 2.5 to 3 cm². Phenotypic characterization of single-cell suspensions generated from freshly isolated tumor material revealed that the pattern of cell-surface expression is identical to that of the injected tumor cells from cell culture. Comparable results were obtained following i.p. inoculation of CX⁺ and CX[−] cells. Macroscopic and microscopic evaluation of lymph nodes, lung, liver and spleen at autopsy of tumor-bearing mice showed no tumor burden except the primary tumor, following s.c. or i.p. injection. After i.v. inoculation of CX⁺ and of CX[−] cells, weak tumor growth was observed in lung and liver, the Hsp70 cell-surface-expression pattern on these tumors being identical to that of the injected cells. However, o.t. injection of colon-carcinoma cell lines CX⁺ and CX[−] into the cecum resulted in tumor growth at the injection site and in spread of distant metastases in lung, liver and spleen. Most interestingly, and in contrast to the primary colon carcinomas, metastases of CX⁺ and of CX[−] tumor cells both revealed strong Hsp70 plasma-membrane expression, although the total amount of cytoplasmic Hsp70 was comparable. *Int. J. Cancer* 77:942–948, 1998.

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Mice with severe combined immunodeficiency (SCID) are lacking in functional, mature T and B cells, due to a defect in the DNA-double-strand-break-repair system that affects the VDJ recombination (Bosma *et al.*, 1983; Schuler *et al.*, 1986; Bosma and Carroll, 1991; Fulop and Phillips, 1990; Lieber *et al.*, 1988; Blunt *et al.*, 1995). SCID/beige mice exhibit even more pronounced immunodeficiency, since they also lack NK cells (Talmadge *et al.*, 1980). For these reasons, SCID/beige mice prove ideal hosts for xenotransplantation. Several reports have described the successful engraftment of human leukemic cell lines (Giavazzi *et al.*, 1995; Cesano *et al.*, 1993; Kamel-Reid *et al.*, 1989), melanoma and solid-tumor cells in immunodeficient mice (Mueller and Reisfeld, 1991; Hill *et al.*, 1994). SCID/beige mice also support the development of distant metastases of certain human tumor cell lines (Reddy *et al.*, 1987; Kamel-Reid *et al.*, 1989; Hill *et al.*, 1991; Cuenca *et al.*, 1996). A step towards more promising therapeutic strategies would be a better understanding of the metastatic process in general. This can be studied in detail in valid experimental metastatic animal model systems. Here, we analyze the influence of Hsp70 membrane expression on tumor incidence and/or development of metastases following different implantation procedures in SCID/beige mice. It is known that most realistic models for tumor metastases can be achieved either by i.v. or by orthotopic (o.t.) implantation of tumor cells (Hoffman, 1994; Kubota, 1994; Fu *et al.*, 1992a,b; Wang *et al.*, 1992). In our system we compared s.c., i.p., i.v. and o.t. inoculation procedures.

We have demonstrated tumor-selective Hsp70 cell-surface expression by immunological and biochemical methods, *in vitro* (Multhoff *et al.*, 1995a,b; Multhoff and Hightower, 1996; Botzler *et al.*, 1996a,b). In contrast to tumor cells, normal cells, including peripheral-blood lymphocytes (PBL), fibroblasts or endothelial cells, lack expression of Hsp70 on their plasma membrane. In the present study, the role of Hsp70 plasma-membrane expression on tumorigenicity and on the capacity to develop distant metastases *in vivo* was analyzed using autologous, clonal sub-lines CX⁺ and CX[−], generated by cell-sorting of CX2 cells. CX⁺ and CX[−] show identical MHC and adhesion-molecule expression patterns (Multhoff *et al.*, 1997), but differ profoundly in their capacity to express Hsp70 on their plasma membrane: CX⁺ cells express Hsp70 stably on more than 90% of the cells, whereas the cell-surface expression of Hsp70 on CX[−] cells was detectable on less than 20% (Multhoff *et al.*, 1997). These cell lines thus provide an ideal tool to study the influence of Hsp70 membrane expression on tumorigenicity and metastases *in vivo*.

MATERIAL AND METHODS

Tumor cell lines

The following carcinoma cell lines, which differ in their capacity to express Hsp70 on their plasma membrane but exhibit identical MHC and adhesion-molecule expression patterns (Multhoff *et al.*, 1997), were cultured at 37°C in 5% CO₂ atmosphere and RPMI-1640 medium (GIBCO, Eggenstein, Germany) supplemented with heat-inactivated 10% FCS (GIBCO) and 2 mM L-glutamine and antibiotics (penicillin/streptomycin). All cell lines were free from mycoplasma contamination, as determined by repeated tests. The cell lines were kept in culture under exponential growth conditions, and harvested with trypsin/EDTA solution. Following counting and washing, the cells were suspended in conditioned RPMI-1640. Only single-cell suspensions of >90% viability, as determined by Trypan-blue-exclusion assays, were used for inoculation into SCID/beige mice.

Mice and tumor-cell inoculation

Pathogen-free female CB-17 *scid/scid* (SCID/beige) mice, 6 to 8 weeks old, were obtained from an animal-breeding colony (Harlan Winkelmann, Borcheln, Germany). The mice were maintained in sterile conditions in micro-isolator cages and given autoclaved food and water. All manipulations were carried out in an air-filtered laminar-flow hood. The mice received s.c., i.p. or i.v. injections into

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the tail vein of 2.5×10^6 exponentially growing tumor cells suspended in 0.1 ml PBS.

For o.t. injection, the animals were anesthetized with pentobarbital (40 mg/kg i.p.) 15 min before surgery. Following a midline incision in the lower abdomen, part of the cecum was carefully taken out of the abdomen and placed onto a wet sterile pad. The o.t. injection was performed outside the abdomen by serial injections ($3 \times 20 \mu\text{l}$ each) of 2.5×10^6 tumor cells suspended in 60 μl PBS, by means of a 30-gauge needle and a 100- μl glass syringe (Hamilton, Bonaduz Switzerland), to diminish the risk of contaminating tumor cells entering the peritoneal cavity. After surgery, the abdomen was closed with staples.

Autopsy

On days 18 and 21 following injection (s.c., i.p., i.v. or o.t.) of tumor cells, the mice were killed by CO_2 . After careful inspection, the primary tumor, lymph nodes, spleen, liver and lungs were measured, collected and cut into pieces. Single-cell suspensions of these organs were obtained by gently teasing the tissue in trypsin/EDTA solution for 60 min at room temperature.

Electron microscopy

Tumor tissue excised from the mice was cut into small pieces (1 mm^3) with a scalpel blade and fixed in 8% paraformaldehyde in HEPES buffer (250 mM) for 4 hr. Free aldehyde groups were quenched with 50 mM NH_4Cl solution. For cryoprotection the tissue was kept in a 2.1 M sucrose solution 17% polyvinylpyrrolidone at 20°C for 30 min. Then the pieces were frozen in liquid nitrogen, and ultrathin sections (70 nm) were cut at -100°C on an Ultracut E microtome (Reichert-Jung FC4E, Vienna, Austria) by a glass knife. Immunogold labeling of Hsp70 was performed with ultra-small gold probes followed by silver enhancement according to Danscher (1981). Briefly, the grids were rinsed and blocked in 0.1% acetylated BSA buffer, then incubated with an anti-Hsp70 monoclonal antibody (MAb) (RPN1197, dilution 1:200; Amersham, Braunschweig, Germany) overnight at 4°C . After washing, the cells were incubated in goat anti-mouse IgG/IgM (Aurion, Cologne, Germany) GP-Ultra small gold in 0.1% acetylated BSA buffer diluted 1:75 for 3 hr at room temperature. Non-specific binding was blocked by extensive washing in 0.1% acetylated BSA buffer. Additional fixation in 2.0% glutaraldehyde in PBS was performed after immunostaining. After silver enhancement, the sections were stained in uranyl acetate/methyl cellulose and viewed in a Zeiss EM 10CR electron microscope. This method is an approach for the detection of antigens localized both in intracellular and in extracellular compartments.

Culture of tumor cells from SCID/beige mice

Solid tumors and organs containing metastases were excised from individual tumor-bearing animals in sterile conditions and disaggregated by scraping the tumor into small slices with a scalpel blade in RPMI-1640 medium containing 1% penicillin/streptomycin solution. After gentle teasing of the tissue in trypsin/EDTA solution for 60 min at room temperature, the material was passed through a sterile wire mesh. Following 2 washings in RPMI 1640, single cells were counted and seeded into small culture flasks and/or directly analyzed by flow-cytometric analysis. The human origin of the tumors and metastases was confirmed by staining with an anti-human-MHC-class-I-specific MAb, W6/32 (Dianova, Hamburg, Germany).

Flow cytometry

Indirect immunofluorescence studies were performed with the use of the Hsp70-specific MAb RPN1197, also an isotype-matched IgG₁-negative control antibody (Immunotech, Marseille, France), human HLA-class-I MAb W6/32, human HLA-DR MAb L243,

ICAM-1 MAb anti-CD54, NCAM MAb anti-CD56, VCAM MAb anti-CD106 or HNK1 MAb anti-CD57 as primary antibodies and a second FITC-conjugated rabbit-anti-mouse antibody (Dako, Hamburg, Germany). The percentage of positively stained cells was determined as the number of positively stained cells minus the number of cells stained with an isotype-matched negative control antibody.

Total Hsp70-expression levels of tumor cells *in vitro*, primary tumors and metastases were compared by flow cytometry using the "Fix and Perm" permeabilization kit (An Der Grub, Kaumberg, Austria) and the Hsp70-specific MAb RPN1197. Relative quantitative measurements of total Hsp70 expression were made by calculating the mean phycoerythrin (PE) fluorescence intensity of cells stained with Hsp70 MAb, normalized to the fluorescence intensity of cells stained with an isotype-matched control antibody (IgG1).

TABLE 1 - TUMORIGENICITY AND METASTASES OF HUMAN COLON-CARCINOMA CELL LINES CX⁻, CX2 AND CX⁺, WHICH DIFFER IN THEIR CAPACITY TO EXPRESS Hsp70 ON THEIR PLASMA MEMBRANE, 18 DAYS AFTER S.C. INJECTION OF 2.5×10^6 TUMOR CELLS INTO SCID/BEIGE MICE¹

Cell lines	CX2	CX ⁺	CX ⁻
Tumor size	3.0	2.6	2.5
Tumor take	11/12	8/8	8/8
Localization	s.c.	s.c.	s.c.
Hsp70-positive cells, %	65	95	24
Metastases	none	none	none

¹Phenotypic characteristics of CX2 colon-carcinoma cell line (Tumorzentrum Heidelberg, TZB 610005, Germany) and the clonal sub-lines CX⁺ and CX⁻ are as shown below. CX2: Hsp70, 61%; ICAM-1, 58%; VCAM, 8%; HNK1, 7%; NCAM, 21%; MHC-I, 99%. CX⁺: Hsp70, 92%; ICAM-1, 60%; VCAM, 10%; HNK1, 11%; NCAM, 24%; MHC-I, 98%. CX⁻: Hsp70, 20%; ICAM-1, 52%; VCAM, 9%; HNK1, 18%; NCAM, 23%; MHC-I, 99%. MHC-II (HLA DR) expression on CX2, CX⁺ and CX⁻ cells was always less than 10%.

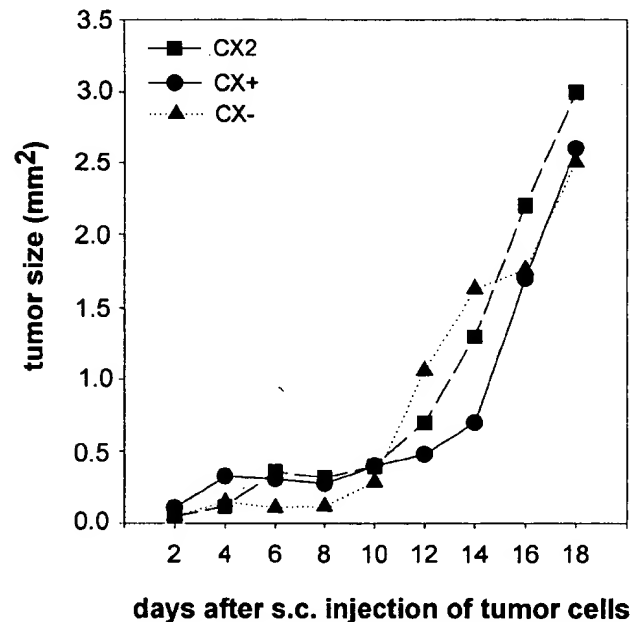


FIGURE 1 - The tumor growth was determined every 2 days by calliper measurement of tumor diameter in 2 dimensions. Mean tumor size of human colon-carcinoma cell lines CX2, CX⁺ and CX⁻, which differ in their capacity to express Hsp70 on their plasma membrane.

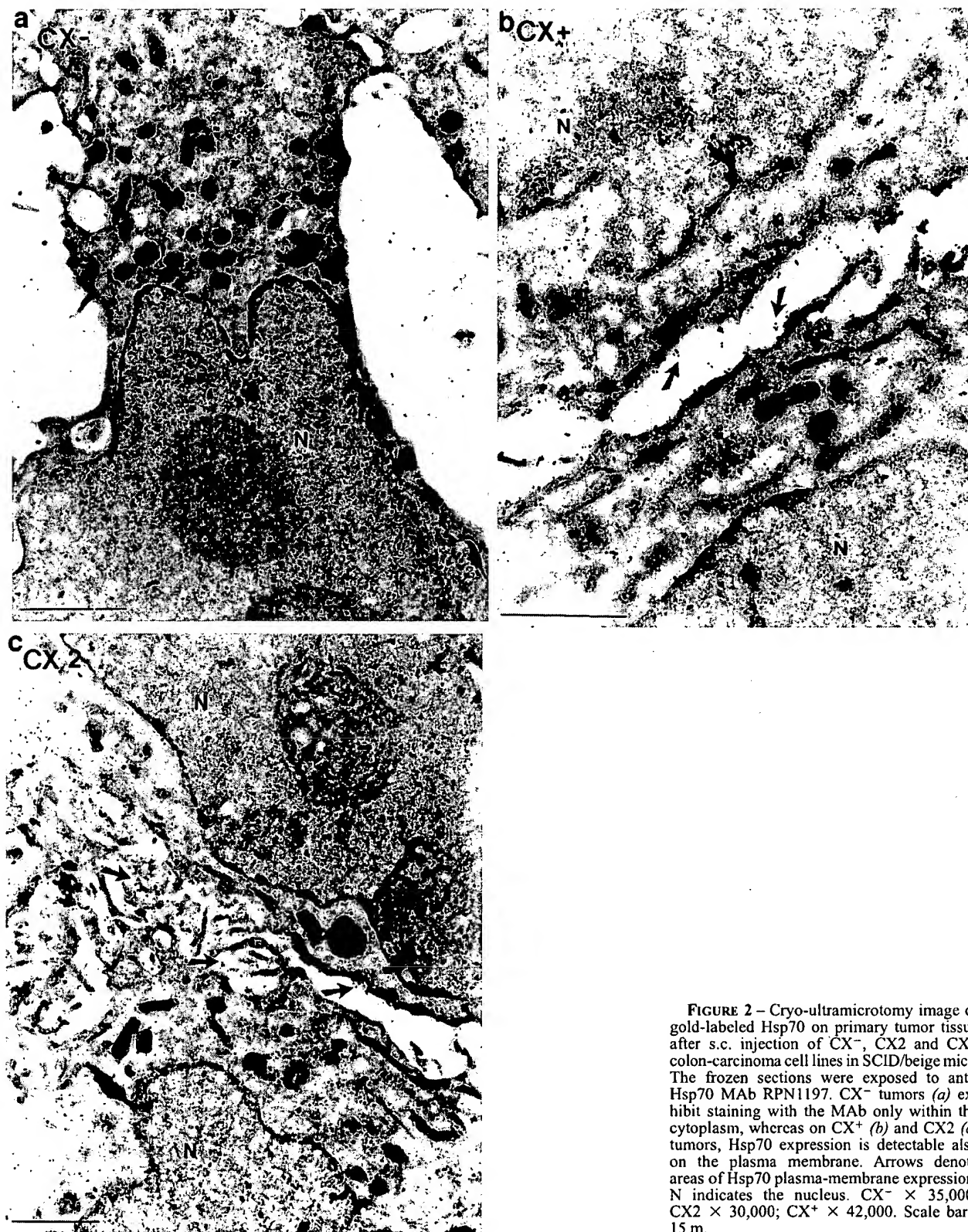


FIGURE 2—Cryo-ultramicrotomy image of gold-labeled Hsp70 on primary tumor tissue after s.c. injection of CX⁻, CX2 and CX⁺ colon-carcinoma cell lines in SCID/beige mice. The frozen sections were exposed to anti-Hsp70 MAb RPN1197. CX⁻ tumors (a) exhibit staining with the MAb only within the cytoplasm, whereas on CX⁺ (b) and CX2 (c) tumors, Hsp70 expression is detectable also on the plasma membrane. Arrows denote areas of Hsp70 plasma-membrane expression. N indicates the nucleus. CX⁻ × 35,000; CX2 × 30,000; CX⁺ × 42,000. Scale bars, 15 μm.

RESULTS

S.c. inoculation of tumor cells with different Hsp70 cell-surface-expression patterns but identical MHC- and adhesion-molecule-expression patterns

Flow-cytometric analysis revealed that CX2, CX⁺ and CX⁻ tumor cell lines exhibit identical MHC-class-I- and adhesion-molecule-expression patterns but differ significantly with respect to their capacity to express Hsp70 on their plasma membrane (Multhoff *et al.*, 1997). As shown in Table I, all tumor cell lines resulted in tumor take in SCID/beige mice when 2.5×10^6 cells were injected s.c. The mean tumor growth, as determined by Caliper measurements of tumor diameter in 2 dimensions every 2 days on viable SCID/beige mice, was comparable for CX2, CX⁺ and CX⁻ tumor cells (Fig. 1). After 18 days, the size of all tumors ranged from 2.5 to 3.0 cm² (Table I). Flow-cytometric analysis of single-cell suspensions of the tumors showed that the cell-surface-expression patterns of the injected tumor cells and the tumors derived from the mice were identical (Table I). Comparable results were obtained with LX-1 lung-carcinoma cells which exhibit plasma-membrane expression on more than 90% of the cells (data not shown). Following s.c. injection, none of the tested tumor cells results in metastatic progression, as determined by macroscopic and microscopic evaluation of different organs including lung, liver, spleen, heart and lymph nodes. X-ray analysis of the skeleton revealed no bone metastases (data not shown).

Electron-microscopical analysis of tumors grown in SCID/beige mice

Cell-surface expression and subcellular distribution of Hsp70, visualized as black dots, was studied on CX⁻, CX2 and CX⁺ tumors by electron microscopy. In line with the data obtained by flow cytometry, only a few tumor cells were found to exhibit Hsp70 plasma-membrane expression when CX⁻ tumor cells were injected (Fig. 2a). Although the amount of cytoplasmic Hsp70 was comparable in CX⁻, CX2 and CX⁺ tumor cells (Multhoff *et al.*, 1997), the number of cells expressing Hsp70 on their plasma membrane was significantly increased when CX2 or CX⁺ tumor cells were injected. Figure 2b shows a representative electron micrograph indicating that all tumor cells express Hsp70 on their plasma membrane after injection of CX⁺ tumor cells. Figure 2c shows 2 neighboring tumor cells with (upper part) and without (lower part) Hsp70 cell-surface expression after injection of CX2 tumor cells.

I.p. and o.t. implantation of CX⁺ and CX⁻ tumor cells in SCID/beige mice

In an attempt to correlate tumorigenicity and metastatic progression with plasma-membrane expression of Hsp70, autologous colon-carcinoma cell lines CX⁺ and CX⁻ that differ in their capacity to express Hsp70 on the plasma membrane *in vitro* were implanted into SCID/beige mice i.p. or o.t. into its site of origin. On the basis of our earlier findings that the tumor take of CX⁺ and CX⁻ cells was about 100% after s.c. injection of 2.5×10^6 tumor cells, the same amount of tumor cells was used for i.p. and o.t. inoculation. In order to support the development of metastases, the mice were killed only on day 21, when signs of severe ill-health, including weight loss and breathing problems, were observed. Following i.p. injection, local tumor growth of a size up to 1.8 cm² was detectable within the intraperitoneal cavity adjacent to the pancreas or within the para-uterine fat tissue. O.t. implantation of tumor cells resulted in local tumor growth adjacent to the injection site within the colon. As shown in Table II, no spread of metastases was detectable in lymph nodes, lungs, spleen or liver following i.p. injection, as determined by macro- and microscopical analysis. However, after o.t. injection with CX⁺ or with CX⁻ tumor cells, nearly all animals developed macroscopical visible metastases of a size up to 0.005 cm² in lung, liver and spleen; lymph nodes were always free from metastases. Due to the relatively small size of the metastases, only qualitative, but no quantitative characterization of the material was possible.

TABLE II - TUMORIGENICITY AND METASTASES OF HUMAN CARCINOMA CELL LINES CX⁺ Hsp70⁺ (90%) AND CX⁻ Hsp70⁺ (20%) THAT DIFFER WITH RESPECT TO THEIR CAPACITY TO EXPRESS Hsp70 ON THEIR PLASMA MEMBRANE 21 DAYS AFTER I.P. OR O.T. INJECTION OF 2.5×10^6 CELLS INTO SCID/BEIGE MICE

Cell lines	21 days after i.p. injection		21 days after o.t. injection	
	CX ⁻	CX ⁺	CX ⁻	CX ⁺
Tumor size	1.8 cm ²	1.5 cm ²	0.5 cm ²	0.4 cm ²
Tumor take	11/13	13/14	12/15	13/13
Localization	i.p. cavity	i.p. cavity	colon	colon
Metastases	none	none	8/12	9/13
Size of metastases	none	none	<0.005 cm ²	<0.005 cm ²
Localization				
Lymph nodes	0/13	0/14	0/8	0/8
Lungs	0/13	0/14	4/12	7/13
Spleen	0/13	0/14	5/12	6/13
Liver	0/13	0/13	5/12	6/13
Hsp70-positive cells, %	none	none	91	85

The human origin of the primary tumors and metastases was confirmed by flow-cytometric analysis using an anti-human-MHC-class-I-specific MAb on single-cell suspensions. The Hsp70 cell-surface-expression pattern was determined directly and/or following cell culture of the tumor material derived from single-cell suspensions. All experiments with single-cell suspensions from cell culture were performed preceding the third cell passage with viable, growing adherent cells. The results of representative flow-cytometric analysis of tumors following i.p. or o.t. injection are shown in Figures 3 and 4. The Hsp70 cell-surface-expression pattern of tumor-cell suspensions of CX⁻ and CX⁺ cells in culture used for injection into SCID/beige mice is shown in the first row; the second row represents Hsp70 plasma-membrane-expression pattern on primary tumor material derived from the intraperitoneal cavity following i.p. injection (Fig. 3) or from the colon following o.t. injection (Fig. 4). The third row in Figure 4 demonstrates a representative phenotypic profile of Hsp70 expression on metastases (spleen). Most interestingly, metastases derived from mice injected with CX⁺ and CX⁻ tumor cells all exhibited strong Hsp70 plasma-membrane expression on more than 85% of the cells (Table II). In contrast to the Hsp70 cell-surface-expression pattern, MHC-class-I and adhesion-molecule expression remained stable in CX⁺ and CX⁻ cells derived from cell culture, from primary tumors and from metastases (data not shown).

We reported earlier that although CX⁺ and CX⁻ tumor cells clearly differ in their capacity to express Hsp70 on their plasma membrane, the cytoplasmic amount of Hsp70 in CX⁺ and CX⁻ cells was identical (Multhoff *et al.*, 1997). Here we compare the cytoplasmic amount of Hsp70 from CX⁺ and CX⁻ cells with single-cell suspensions derived from primary tumors and metastases following o.t. injection in SCID/beige mice. As shown in Table III, we observed no significant difference in the total cytoplasmic amount of Hsp70 as determined by flow-cytometric analysis using permeabilized cells.

I.v. implantation of CX⁺ and CX⁻ tumor cells in SCID/beige mice

Compared with the s.c. and i.p. injection procedures, the tumor-take rate and the average tumor size were significantly lower following i.v. injection (0.3 cm²), when 2.5×10^6 tumor cells were injected (Table IV). Furthermore, following i.v. injection tumor burden was found only in lungs and liver, not in any other organs. The results from flow-cytometric analysis derived from single-cell suspensions of tumor material after i.v. injection from the lung and liver are shown in Table IV. In contrast to the results obtained with lung metastases after o.t. injection, lung tumors after i.v. injection of CX⁻ cells remained Hsp70-negative; after injection of CX⁺ cells the resulting tumors were Hsp70-positive.

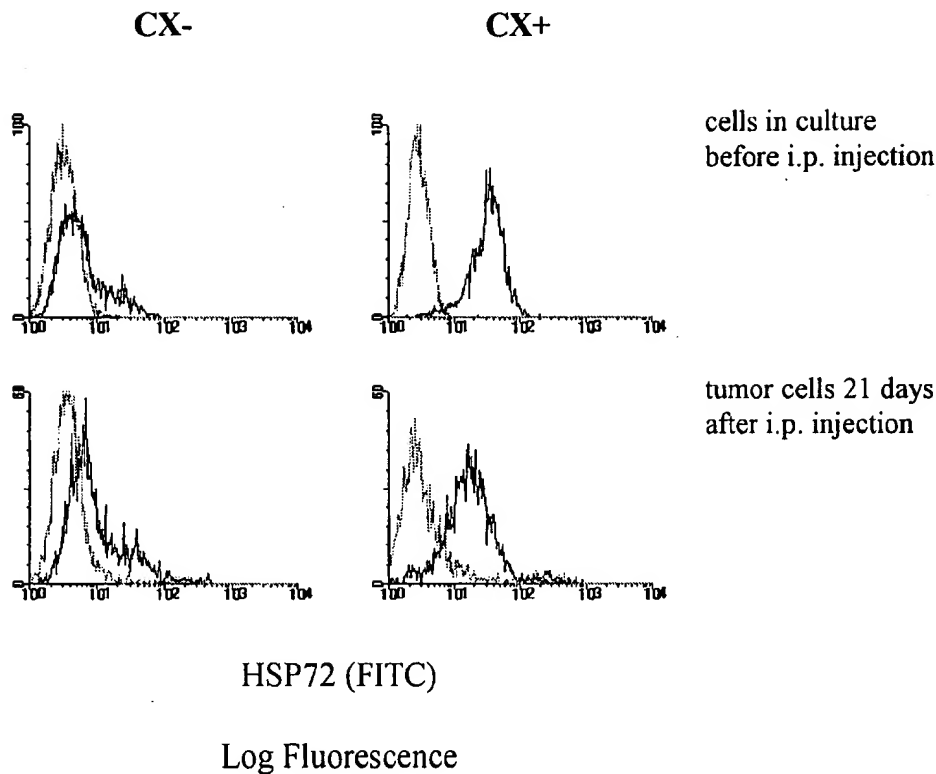


FIGURE 3 – Hsp70-flow-cytometric profile of CX⁻ and CX⁺ tumor cells in culture before injection (upper row) compared with that of single-cell suspensions derived from primary tumor tissue of SCID/beige mice 21 days after i.p. injection (second row). Results are expressed as log green fluorescence intensity vs. relative cell numbers. Dotted line, background staining with an isotype-matched negative control antibody; solid line, Hsp70 cell-surface expression.

DISCUSSION

The limited availability of metastatic animal models for human tumors is still a barrier in understanding the process leading to tumor progression and metastatic development. The s.c. and i.p. injection of human carcinoma cells into immunodeficient mice rarely results in metastases (Ware *et al.*, 1982). Our data confirm these findings: following s.c. or i.p. injection of colon-carcinoma sub-lines, we observed local tumor growth without spread of metastases. Initial tumor growth was measurable as little as 2 days post-injection, indicating rapid tumor progression.

In the present study we wished to analyze the role of Hsp70 plasma-membrane expression on tumorigenicity and spread of metastases. We therefore injected into SCID/beige human colon-carcinoma sub-lines that differ significantly, *in vitro*, in their capacity to express Hsp70 on their cell membrane, but exhibit identical MHC- and adhesion-molecule-expression patterns. Membrane expression of Hsp70 appears not to be relevant for tumorigenicity, since the tumor growth rates of CX⁻, CX2 and CX⁺ were not significantly different. In addition, the cell-surface-expression pattern of Hsp70 on tumor biopsy material of primary tumors derived from SCID/beige mice following s.c. and i.p. injection was comparable with that of the injected cell lines. Cell-surface expression of MHC or adhesion molecules has been shown to play a role in tumorigenicity and metastatic growth (Bao *et al.*, 1993). However, in our system, involvement of these molecules could be excluded, since the autologous tumor sub-lines CX⁺ and CX⁻ exhibit identical MHC- and adhesion-molecule-expression patterns *in vitro* (Multhoff *et al.*, 1997) and, *in vivo*.

In order to evaluate the potential role of Hsp70 expression on metastatic progression, an orthotopic tumor engraftment was

performed. The inoculation of vital tumor cells into their site of origin has been shown to be effective in inducing rapid local tumor growth and metastatic disease (Fidler, 1986; Fu *et al.*, 1992a,b). In our system, the human colon carcinoma sub-lines CX⁺ and CX⁻ were injected into the cecum of SCID/beige mice. After o.t. engraftment of these 2 sub-lines, we observed no significant differences in the localization of metastases, which were found in lung, spleen and liver; lymph nodes always remained tumor-free. Due to the small size of metastases, only phenotypic characterization of single-cell metastases was possible. Most interestingly, metastases derived after o.t. injection of CX⁺ or CX⁻ tumor cells always exhibited strong Hsp70 membrane expression. As with the primary tumors, MHC and adhesion-molecule expression remained unaltered. These data led us to speculate that Hsp70 might be involved in the process of metastases in immunodeficient mice.

It has been reported that, in addition to o.t. injection, i.v. inoculation of tumor cells supports the generation of distant metastases (Taniguchi *et al.*, 1987; Talmadge *et al.*, 1980). To determine whether Hsp70 cell-surface expression facilitates migration and dissemination of metastases or whether Hsp70-positive cells have a better chance to invade and/or grow in lungs, liver or spleen, i.v. injection was performed using CX⁺ and CX⁻ sub-lines. In contrast to metastases derived after o.t. injection, tumor cells derived from the lung and liver following i.v. injection revealed no increased Hsp70 cell-surface expression. Together with the finding that tumor take as well as tumor growth was much weaker following i.v. injection as compared with other injection procedures, these data indicate that Hsp70 cell-surface expression is more likely involved in the escape of tumor cells from the primary

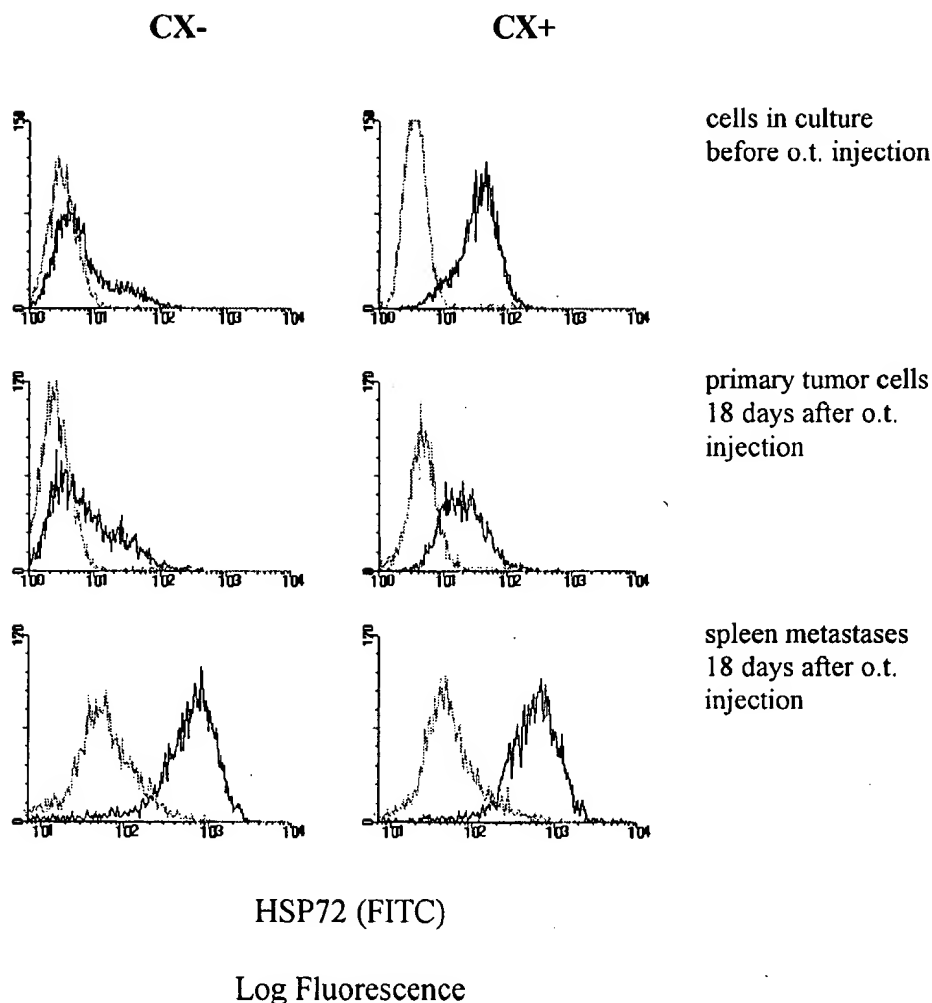


FIGURE 4 – Hsp70-flow-cytometric profile of CX⁻ and CX⁺ tumor cells in culture before injection (upper row) compared with that of single-cell suspensions derived from primary tumor tissue of SCID/beige mice 21 days after o.t. injection (second row) and that of metastatic tissue (spleen; third row). Results are expressed as log green fluorescence intensity vs. relative cell numbers. Dotted line, background staining with an isotype-matched negative control antibody; solid line, Hsp70 cell-surface expression.

TABLE III – RELATIVE AMOUNT OF TOTAL Hsp70 IN PRIMARY TUMORS AND LUNG METASTASES DERIVED AFTER O.T. INJECTION OF CX⁺ AND CX⁻ TUMOR CELLS INTO SCID/BEIGE MICE COMPARED WITH CX⁺ AND CX⁻ TUMOR CELLS *IN VITRO*

Tumor cells derived from	Relative amount of total Hsp70	
	CX ⁺ cells	CX ⁻ cells
primary tumors after o.t. injection of CX ⁺ tumor cells	1.08	0.99
primary tumors after o.t. injection of CX ⁻ tumor cells	1.09	1.22
lung metastases after o.t. injection of CX ⁺ tumor cells	0.98	1.09
lung metastases after o.t. injection of CX ⁻ tumor cells	0.90	1.00

tumors than in the capacity to enhance invasion and growth in lung or liver.

In vitro results using Hsp70-positive tumor cells in a standard chromium-51-release cytotoxicity assay revealed that sensitivity to lysis of CX⁺ single-cell suspensions of metastases and CX⁺ cells

TABLE IV – TUMORIGENICITY OF HUMAN CARCINOMA CELL LINES CX⁺ AND CX⁻ THAT DIFFER IN THEIR CAPACITY TO EXPRESS Hsp70 ON THEIR PLASMA MEMBRANE FOLLOWING I.V. INJECTION OF 2.5×10^6 CELLS INTO THE TAIL VEIN OF SCID/BEIGE MICE

Cell lines	CX ⁻	CX ⁺
Hsp70-positive cells before injection, %	22	94
Days post-i.v. injection	21	21
Tumor size	0.2 cm ²	0.15 cm ²
Tumor take	3/4	2/4
Localization	lung	lung/liver
Hsp70-positive cells after injection, %	23	89

of primary tumors was not significantly different (data not shown). It has been demonstrated that certain cytoplasmic localized heat-shock proteins, including Hsp70, are expressed on tumor cells, but not on normal tissue (Multhoff *et al.*, 1995a), thus indicating that HSP might elicit tumor-specific immunity (Piselli *et al.*, 1995; Poccia *et al.*, 1992; Ferrarini *et al.*, 1992; Chouchane *et al.*, 1994; Fisch *et al.*, 1992; Botzler *et al.*, 1996b). We have shown that cell-membrane-expressed Hsp70 acts as an immunological target structure for recognition by NK cells (Multhoff *et al.*, 1995a,b

1997; Botzler *et al.*, 1996a,b). Taken together these data led us to the hypothesis that NK cells with specificity against Hsp70 expressing tumor cells might not only result in an improved local tumor control but also might reduce the spread of distant metastases. This would be in line with the results of Hill *et al.* (1994), who showed that human NK cells possess the ability to mediate anti-tumor activity and to reduce lung-nodule counts of human melanoma xenografts. Studies continue to investigate the role of

human NK cells transferred into tumor-bearing SCID/beige mice in protection against Hsp70-expressing tumor cells and metastases.

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REFERENCES

- BAO, L., PIGOTT, R., MATSUMURA, Y., BABAN, D. and TARIN, D., Correlation of VLA-4 integrin expression with metastatic potential in various human tumour cell lines. *Differentiation*, **52**, 239–246 (1993).
- BLUNT, T., FINNIE, N.J., TACCIOLI, G.E., SMITH, G.C., DEMENGEOT, J., GOTTLIEB, T.M., MIZUTA, R., VARGHESE, A.J., ALT, F.W. and JEGGO, P.A., Defective DNA-dependent protein-kinase activity is linked to V(D)J recombination and DNA-repair defects associated with the murine SCID mutation. *Cell*, **80**, 813–823 (1995).
- BOSMA, G.C., CUSTER, R.P. and BOSMA, M.J., A severe-combined-immunodeficiency mutation in the mouse. *Nature (Lond.)*, **301**, 527–530 (1983).
- BOSMA, M.J. and CARROLL, A.M., The SCID mouse mutant, definition, characterization, and potential uses. *Ann. Rev. Immunol.*, **9**, 323–350 (1991).
- BOTZLER, C., ISSELS, R. and MÜLTHOFF, G., Heat-shock-protein-72 cell-surface expression on human lung-carcinoma cells is associated with an increased sensitivity to lysis mediated by adherent natural-killer cells. *Cancer Immunol. Immunother.*, **43**, 226–230 (1996a).
- BOTZLER, C., KOLB, H.J., ISSELS, R.D. and MÜLTHOFF, G., Non-cytotoxic alkyl-lysophospholipid treatment increases sensitivity of leukemic K562 cells to lysis by natural-killer cells (NK). *Int. J. Cancer*, **65**, 633–638 (1996b).
- CESANO, A., O'CONNOR, R., NOWELL, P.C., LANGE, B., CLARK, S.C. and SANTOLI, D., Establishment of a karyotypically normal cytotoxic leukemic T-cell line from a T-ALL sample engrafted in SCID mice. *Blood*, **81**, 2714–2722 (1993).
- CHOUGHANE, L., BOWERS, S., SAWADIKOSOL, S., SIMPSON, R.M. and KINDT, T.J., Heat-shock proteins expressed on the surface of human T-cell-leukemia-virus-Type-1-infected cell lines induce auto-antibodies in rabbits. *J. infect. Diseases*, **169**, 253–259 (1994).
- CUENCA, R.E., TAKITA, H. and BANKERT, R., Orthotopic engraftment of human lung tumours in SCID mice for the study of metastasis. *Surg. Oncol.*, **5**, 85–91 (1996).
- DANSCHER, G., Localization of gold in biological tissue, a photochemical method for light and electron microscopy. *Histochemistry*, **71**, 81–87 (1981).
- FERRARINI, M., HELTAI, S., ZOCCHI, M.R. and RUGARLI, C., Unusual expression and localization of heat-shock proteins in human tumor cells. *Int. J. Cancer*, **51**, 613–619 (1992).
- FIDLER, I.J., Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. *Cancer Metastasis Rev.*, **5**, 29–49 (1986).
- FISCH, P., OETTEL, K., FUDIM, N., SURFUS, J.E., MALKOVSKY, M. and SONDEL, P.M., MHC-unrestricted cytotoxic and proliferative responses of two distinct human gamma/delta T-cell sub-sets to Daudi cells. *J. Immunol.*, **148**, 2315–2323 (1992).
- FU, X., GUADAGNI, F. and HOFFMAN, R.M., A metastatic nude-mouse model of human pancreatic cancer constructed orthotopically with histologically intact patient specimens. *Proc. nat. Acad. Sci. (Wash.)*, **89**, 5645–5649 (1992a).
- FU, X., HERRERA, H., KUBOTA, T. and HOFFMAN, R.M., Extensive liver metastasis from human colon cancer in nude and SCID mice after orthotopic implantation of histologically intact human colon-carcinoma tissue. *Anticancer Res.*, **12**, 1395–1397 (1992b).
- FULOP, G.M. and PHILLIPS, R.A., The SCID mutation in mice causes a general defect in DNA repair. *Nature (Lond.)*, **347**, 479–482 (1990).
- GIACAZZI, R., DI BERARDINO, C., GAROFALO, A., MOTTA, T., GOBBI, A., SCANZIANI, E., GIUDICI, G., GALLI, M., MAYO, J.G., BIONDI, A. and RAMBALDI, A., Establishment of human acute-myelogenous-leukemia lines secreting interleukin-1 beta in SCID mice. *Int. J. Cancer*, **61**, 280–285 (1995).
- HILL, L.L., KORNGOLD, R., JAWORSKY, C., MURPHY, G., MCCUE, P. and BERD, D., Growth and metastasis of fresh human melanoma tissue in mice with severe combined immunodeficiency. *Cancer Res.*, **51**, 4937–4941 (1991).
- HILL, L.L., PERUSSIA, B., MCCUE, P.A. and KORNGOLD, R., Effect of human natural-killer cells on the metastatic growth of human melanoma xenografts in mice with severe combined immunodeficiency. *Cancer Res.*, **54**, 763–770 (1994).
- HOFFMAN, R.M., Orthotopic is orthodox, why are orthotopic-transplant metastatic models different from all other models? *J. cell. Biochem.*, **56**, 1–3 (1994).
- KAMEL-REID, S., LETARTE, M., SIRARD, C., DOEDENS, M., GRUNBERGER, T., FULOP, G., FREEDMAN, M.H., PHILLIPS, R.A. and DICK, J.E., A model of human acute lymphoblastic leukemia in immune-deficient SCID mice. *Science*, **246**, 1597–1600 (1989).
- KUBOTA, T., Metastatic models of human cancer xenografted in the nude mouse, the importance of orthotopic transplantation. *J. cell. Biochem.*, **56**, 4–8 (1994).
- LIEBER, M.R., HESSE, J.E., LEWIS, S., BOSMA, G.C., ROSENBERG, N., MIZUUCHI, K., BOSMA, M.J. and GELLERT, M., The defect in murine severe combined immune deficiency, joining of signal sequences but not coding segments in V(D)J recombination. *Cell*, **55**, 7–16 (1988).
- MUELLER, B.M. and REISFELD, R.A., Potential of the SCID mouse as a host for human tumors. *Cancer Metastasis Rev.*, **10**, 193–200 (1991).
- MÜLTHOFF, G., BOTZLER, C., JENNEN, L., SCHMIDT, J., ELLWART, J. and ISSELS, R., Heat-shock protein 72 on tumor cells: a recognition structure for natural-killer cells. *J. Immunol.*, **158**, 4341–4350 (1997).
- MÜLTHOFF, G., BOTZLER, C., WIESNET, M., EISSNER, G. and ISSELS, R., CD3 large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72-kD heat-shock protein on human sarcoma cells. *Blood*, **86**, 1374–1382 (1995a).
- MÜLTHOFF, G., BOTZLER, C., WIESNET, M., MÜLLER, E., MEIER, T., WILMANN, W. and ISSELS, R.D., A stress-inducible 72-kDa heat-shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. *Int. J. Cancer*, **61**, 272–279 (1995b).
- MÜLTHOFF, G. and HIGHTOWER, L.E., Cell-surface expression of heat-shock proteins and the immune response. *Cell Stress Chaperones*, **1**, 167–176 (1996).
- PISELLI, P., VENDETTI, S., POCCIA, F., CICCONI, R., MATTEI, M., BOLOGNESI, A., STIRPE, F. and COLIZZI, V., *In vitro* and *in vivo* efficacy of heat-shock-protein-specific immunotoxins on human tumor cells. *J. biol. Regul. homeost. Agents*, **9**, 55–62 (1995).
- POCCIA, F., PISELLI, P., DI CESARE, S., BACH, S., COLIZZI, V., MATTEI, M., BOLOGNESI, A. and STIRPE, F., Recognition and killing of tumour cells expressing heat-shock protein 65 kD with immunotoxins containing saporin. *Brit. J. Cancer*, **66**, 427–432 (1992).
- REDDY, S., PICCIONE, D., TAKITA, H. and BANKERT, R.B., Human lung-tumor growth established in the lung and subcutaneous tissue of mice with severe combined immunodeficiency. *Cancer Res.*, **47**, 2456–2460 (1987).
- SCHULER, W., WEILER, I.J., SCHULER, A., PHILLIPS, R.A., ROSENBERG, N., MAK, T.W., KEARNEY, J.F., PERRY, R.P. and BOSMA, M.J., Rearrangement of antigen-receptor genes is defective in mice with severe combined immune deficiency. *Cell*, **46**, 963–972 (1986).
- TALMADGE, J.E., MEYERS, K.M., PRIEUR, D.J. and STARKEY, J.R., Role of NK cells in tumour growth and metastasis in beige mice. *Nature (Lond.)*, **284**, 622–624 (1980).
- TANIGUCHI, K., PETERSSON, M., HOGLUND, P., KIESSLING, R., KLEIN, G. and KARRE, K., Interferon gamma induces lung colonization by intravenously inoculated B16 melanoma cells in parallel with enhanced expression of class-I-major-histocompatibility-complex antigens. *Proc. nat. Acad. Sci. (Wash.)*, **84**, 3405–3409 (1987).
- WANG, X., FU, X. and HOFFMAN, R.M., A new patient-like metastatic model of human lung cancer constructed orthotopically with intact tissue via thoracotomy in immunodeficient mice. *Int. J. Cancer*, **51**, 992–995 (1992).
- WARE, J.L., PAULSON, D.F., MICKY, G.H. and WEBB, K.S., Spontaneous metastasis of cells of the prostate-carcinoma cell line PC-3 in athymic nude mice. *J. Urol.*, **128**, 1064–1067 (1982).

Natural killer cell reactivity: activation and cytolysis mechanism models, involving heat shock protein, haemopoietic histocompatibility, major histocompatibility complex and complement molecules

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Abstract — The close association of heat shock protein (HSP), haemopoietic histocompatibility (Hh), major histocompatibility complex (MHC), and complement genes on the same chromosomal region, and the fact that all these genes are inherited on the whole in each haplotype of an individual, might indicate some evolutionary and functional correlations among them. Several data suggest for HSP70 molecules a possible role as a molecular target recognizable by natural killer (NK) cells. HSP70 sequences from both prokaryotic and eukaryotic organisms reveal that about half of the amino acid residues are identical and many of the remaining residues are similar. I here assume that NK reactivity might start, early in the immunogenesis process, as a effect of the interaction between HSP70 molecules and a hypothetical HSP receptor of yet immature non-cytolytic NK cells. To this receptor, an HSP molecule might act as an activator or an inhibitor depending on whether its amino acid residues are reactive or not with it, respectively. Later in the immunogenesis process, murine Hh or human equivalent molecules, dominantly expressed in bone marrow target cells, might select the non-reactive NK clones of an individual, inducing them to mature and express a lytic machinery. As a consequence of the NK maturation, proliferating hemopoietic target cells expressing only or mainly activator HSPs on their surface might undergo NK cytolysis. This might explain the NK lysis of apparently normal cells found in human foetal marrow; moreover, this might explain in some way the F1 hybrid resistance phenomenon. The NK reactivity of an individual would be further modulated by the expression on the NK surface of particular receptors (CD94, p58) specific for defined MHC molecules (Cw1, Cw3, Bw6, B7) on the target cells. Such a specific interaction would induce an 'NK effector inhibition'. The NK reactivity mechanism might have been further evolutionarily modified and adapted by the involvement of other NK receptors, such as CD11b (specific for the C3b factor of the complement) and CD16 (specific for the IgG Fc piece).

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Cooperation among HSP, MHC, CD11b, CD16, C3b and Fc allows us to propose original models of the activation and cytotoxicity mechanisms in the NK cytotoxicity and antibody-dependent cell cytotoxicity phenomena.

Introduction

Natural killer (NK) cells lyse tumour or virus-stressed cells without restriction by the major histocompatibility complex (MHC) antigens (1). NK cells also recognize and lyse normal allogeneic T-cell blasts (2) and embryonal cells (3). Target molecules recognized by NK cells, and NK receptors for recognition, are, as yet, little known. Some specificities (NK-1, NK-2 and others) in the recognition of normal allogeneic target cells have been shown by Ciccone et al (4). NK specificities may be equivalent to the MHC-linked haemopoietic histocompatibility (Hh) antigens (5), defined by bone marrow grafting in mice (6). Recent reports (7-9) indicate that heat shock protein (HSP) molecules may be involved in the NK reactivity, as I have previously postulated (10). On the basis of these and other data, I suggest an original model of NK reactivity involving HSP, Hh, MHC and complement molecules, whose genes are closely located in the same chromosomal region (5). This close association and the fact that all these genes are inherited on the whole in each haplotype of an individual (11) might indicate that some evolutionary and functional correlation might exist among them. I therefore postulate that: (a) HSP70 molecules might be the natural target recognized by NK cells on proliferating cells; (b) the murine Hh (or human equivalent) molecules might dominantly determine maturation and selection of specific NK clones in the bone marrow of an individual; (c) HLA-B and C molecules, when present on target cells, would modulate the reactivity of the selected NK specificities, by an 'effector inhibition' (12) mechanism.

Development of the hypothesis

Heat shock protein molecules as targets for the basic natural killer cell reactivity

HSP72, a stress-inducible sarcoma molecule, is recognized by CD3- NK cells, but not CD3+ T cells (7). An oxidative stress protein is a factor enhancing NK cells (13). The sensitivity of certain tumour target cells to natural killing increases after stressing (heating) (9), which induces high expression of HSPs. A 70 kDa heat shock cognate (HSC) protein, a rat fibrosarcoma antigen, may act as a presenting molecule (8) for NK cells. HSP70 closely resemble MHC molecules in structure and function (14), and com-

puterized models show on HSP70 peptides binding sites analogous to those present on MHC class I molecules (14). Each of the HSPs appears to interact with many different proteins (15). All this suggests for HSP70 molecules a possible role of molecular target recognized by NK cells, to which HSP70 might present peptides. Comparison of HSP70 sequences from both prokaryotic and eukaryotic organisms reveals that about half of the amino acid residues are identical, and many of the remaining residues are similar; HSP90 and HSP60 are similarly highly conserved (15). Considering these HSP amino acid sequences, I postulate that NK cell reactivity might result as an effect of the interaction between HSP70 molecules and a hypothetical HSP receptor (asialo GM1?, CD2?) of NK cells. This HSP receptor might be endowed with an inhibitor binding site (i-site) and an activator site (a-site). The i-site might be specific for the common amino-acidic sequences of HSP70 molecules; the a-site might have a wide reactivity for the similar or different amino-acidic sequences of the HSP70 family members. Thus, an HSP might react as an inhibitor (i-HSP) or an activator (a-HSP) of the NK reactivity when its non-common amino-acidic sequences are respectively non-reactive or reactive with the a-site of HSP receptors. Since HSP70 are probable peptide presenting molecules (8,14), the presence of other antigens bound on them (15) might alter their stereochemical structure and thus enhance their reactivity with the a-site of NK HSP receptors. The basic NK cell reactivity might consist in an internal signal that mobilizes Ca^{++} ions (16) and switches on the replication, transcription and lytic machinery.

Haemopoietic histocompatibility antigen selection, restriction and maturation of natural killer clones

This basic NK reactivity might presumably start during early immunogenesis, but lytic machinery effectiveness might not occur in yet-immature NK cells. Probably, NK cell maturation and differentiation occur in later phases of the immunogenesis, by a target-cell-mediated pathway (17). I think that NK maturation might be mediated by haemopoietic target cells expressing on their surface murine Hh or human equivalent molecules. Hh molecules are dominantly expressed on bone marrow cell surfaces and are specifically recognized by NK cell receptors in the rejection of bone marrow cell grafts (6). I

suggest that Hh receptors might be associated to the HSP receptors on the NK cell surface, so that the reactivity between a-HSPs and the a-site of HSP receptors might be modulated in the presence of bone marrow target cells endowed with Hh antigens. Moreover, Hh receptor expression or other Hh-related events might induce the ability to express an effective lytic machinery in the immature NK cells, thus rendering them cytolytic, that is mature. As a consequence of the NK maturation, proliferating haemopoietic target cells expressing only or mainly a-HSPs on their surface might undergo NK cytotoxicity. The NK lysis of apparently normal cells found in human foetal marrow (18) might be a sign of this. On the contrary, bone marrow target cells expressing only i-HSPs, or both a-HSPs and i-HSPs, might avoid NK cytotoxicity. Such an NK reactivity model might in some way explain why F1 hybrid mice reject bone marrow cells of both parents (hybrid resistance), whereas hybrid bone marrow cells are accepted by both parents (6). I think that different possible combinations between Hh antigens (specific or not for the NK Hh receptors) and a-HSPs (prevalent or not over i-HSPs) on the target cell surface might account for the said phenomena peculiar to haemopoietic immunity.

Natural killer effector inhibition induced by major histocompatibility complex molecules of target cells

NK reactivity would be further modulated during the ontogenetic differentiation phase by the expression of particular MHC molecules (Cw1, Cw3, Bw6, B7) on the target cells and of specific receptors on the NK surface (CD94, p58) (5,16,19–21). I postulate that these receptors for MHC antigens might be equivalent to the Hh receptors (5) for Hh antigens, and that they also might associate to the HSP receptors. So, they might modulate the reaction between the a-site of the latter and a-HSPs, when MHC antigens are expressed on the target surface. Such a modulation might inhibit, stimulate or have no effect on the basic NK reactivity, depending on the MHC antigens expressed on the target. Specific MHC molecules would interact with specific receptors: CD94 NK molecules seem to be involved in the recognition of HLA-B target antigens, and p58 NK molecules in that of HLA-C (16,20). NK effector inhibition (12) might therefore be due to a specific masking effect by MHC molecules on specific NK receptors associated to the a-site of HSP-receptors. Specific MHC alleles would thus restrict the basic NK reactivity so that autologous proliferating normal cells, even if a-HSP-endowed, might elude NK cytotoxicity.

Further modulation of the natural killer cell reactivity

The NK reactivity mechanism might have been further evolutionarily modified and adapted by the involvement of other NK receptors, such as CD11b receptors and CD16 receptors (22). CD11b receptors are specific for the C3b factor of the complement system (22); they might therefore have a functional homology with the C4b–C2a–B complex of the complement alternate pathway. CD16 receptors are specific for the IgG Fc piece (22); therefore they might have a functional homology with the C1qrs complex of the classic complement pathway. Perforin, a lytic factor of the NK cytoplasmic granules, is homologous to the terminal factors (23) (C8, C9) of the complement. All this strongly suggests that in the NK membrane there might be an embedded complement system, by which the cytolytic activity might occur.

Activation mechanism model in natural killer cell cytotoxicity and antibody-dependent cell cytotoxicity

NK cytotoxicity is typically expressed by NK cells very rich in CD11b receptors (22) specific for C3b. C2, C4 and B complement factors may form a C4b–C2a–B complex binding C3b (24), thus exhibiting a functional homology with CD11b. C2, C4 and B genes are closely associated to HSP70 genes (5). HSP70 molecules may have a role of presenting peptides (8,14). Each of the HSPs appears to interact with many different proteins (15). I suggest, therefore, that target HSP70 might act as a molecule presenting the C3b peptide to the CD11b receptors of NK cells. C3b is largely produced during in-vivo immune processes and commonly binds itself to target cell surfaces, opsonizing them (24). C3b-opsonized cells tend to adhere to the leukocytes (24). In the cytoplasmic granules of NK cells a lytic protein, the so-called 'perforin', is present, which is significantly homologous and equivalent to the complement C8 and C9 factors (23). Cytolytic effects of NK lysis produce perforating lesions 10–20 nm in diameter, very similar to those generated by the C5–6–7–8–9 activated complex of the complement (24). Since CD11b has a functional homology with the C4b–C2a–B complex of the complement alternate pathway, after a CD11b–C3b specific bond the subsequent C5, C6, C7, C8 complement factors might be activated. Once activated, the C5–6–7–8 complex might detach itself from the NK membrane, remaining adherent (24) through C3b–HSP to the target membrane, where it might produce small lesions. At the same time, the C3b–CD11b reaction might

generate an internal signal that mobilizes Ca^{++} ion and alters the NK membrane permeability, thus allowing the external release of the cytolytic granule content (25), such as perforin, fragmentin, TNF or other lytic factors. The said mechanism, evolutionarily modified and adapted, for instance by embedding a C1qrs (or equivalent) preformed complex in the NK membrane, might be used by NK cells in antibody-dependent cell cytotoxicity (ADCC). Since the CD16 receptors are functionally equivalent to the complement C1qrs complex, NK cells rich in CD16 receptors might utilize the aforesaid activation mechanism when the target is sensitized by antibodies. In fact, the Fc piece of target-bound IgG might specifically react with NK CD16 receptors. This Fc-CD16 complex (functionally equivalent to C1qrs), together with CD11b receptors (functionally equivalent to C4b-C2a-B), might allow the C3 factor to be bound and, then, cleaved into C3a and C3b. C3b might activate the C5-6-7-8 complex, as suggested above.

Natural killer cell cytolytic mechanism model

Either NK cytotoxicity or ADCC activation might therefore result in a membrane C5-6-7-8 (or equivalent) activated complex. This, detaching itself from NK membrane, would remain adherent to the target cell surface (24) through C3b-HSPs or Fc of bound IgG. Since such an activated complex is able to cause a slow target lysis (24), some membrane damage (presumably pore formation) might occur, which alters the target membrane permeability. So, exocellular Ca^{++} ions may persistently flow inside the target, inducing the lytic processes of apoptosis and/or necrosis. Theoretically, NK cytolysis might therefore occur through the only C5-6-7-8 activated complex, as perhaps it occurs in the case of rapid necrotic death independent of granule exocytosis (26). However, this basic cytolytic mechanism might be highly enhanced by the presence of lytic factors, such as perforin, fragmentin, RNK-I protease, granzyme B and TNF, present in the NK granules and secreted by exocytosis in the microenvironment between NK and target membranes. Therefore, target lysis may occur with many types of lytic factors or even without exocytosis of lytic factors, and in different ways (apoptosis and/or necrosis) (26-29). This might indicate that the crucial point in the cytolysis might be the formation of few small initial lesions in the target membrane, such as these generated by the C5-6-7-8 activated complex. The membrane damages and the consequent persistent influx of Ca^{++} ions into the target cytoplasm might then massively activate the transcription and proliferation machinery genes (myc, fos, p53, ras) and enzymes (polymerases, endo-

nucleases, kinases) (30). Such a massive prolonged cell machinery activation might be mainly responsible for the apoptotic and/or necrotic target cell death.

References

1. Herberman R B, Ortaldo J R. Natural killer cells: their role in defenses against disease. *Science* 1981; 214: 24-30.
2. Ciccone E, Viale O, Pende D et al. Specific lysis of allogeneic cells after activation of CD3- lymphocytes in mixed lymphocyte culture. *J Exp Med* 1988; 168: 2403.
3. Stern P, Gidlund M, Kimura A, Gronvik K O, Wigzell H. Murine embryonal carcinoma cells: universal targets for mammalian NK cells. *Int J Cancer* 1981; 27: 679-688.
4. Ciccone E, Pende D, Viale O et al. Involvement of HLA class I alleles in Natural Killer (NK) cell-specific functions: expression of HLA-Cw3 confers selective protection from lysis by alloreactive NK clones displaying a defined specificity (specificity 2). *J Exp Med* 1992; 176: 963-971.
5. Colonna M, Spies T, Strominger J L et al. Alloantigen recognition by two human natural killer clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci USA* 1992; 89: 7983-7985.
6. Rembecki R M, Kumar V, David C S, Bennett M. Bone marrow cell transplants involving intra-H-2 recombinant inbred mouse strains. *J Immunol* 1988; 141: 2253-2260.
7. Multhoff G, Botzler C, Wiesnet M, Eissner G, Issels R. CD3- large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72-KD heat shock protein on human sarcoma cells. *Blood* 1995; 86: 1374-1382.
8. Tamura Y, Tsuboi N, Sato N, Kikuchi K. 70 KDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. *J Immunol* 1993; 151(10): 5516-5524.
9. Scott J, Dawson J R. The sensitivity of certain tumour target cells to natural killing increases after stressing (heating). *FASEB J* 1991; 5: A631.
10. Manzo G. Cancer: a biological problem without any natural immunological solution? A unified theory with implications for grafts, pregnancy and tumor immunoprophylaxis. *Med Hypotheses* 1995; 45: 317-324.
11. Clerici E. Il maggiore complesso di Istocompatibilità. In: La Nuova Immunologia. Milano: Le Scienze SpA Editore, 1992: 144-154.
12. Ljunggren H G, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology Today* 1990; 11(7): 237-243.
13. Butterfield L H, Kim A, Shau H. Natural killer enhancing factor is an oxidative stress protein. *Proc Annu Meet Am Assoc Cancer Res* 1994; 35: A3503.
14. Welch W J. Come le cellule reagiscono allo stress. *Le Scienze* (Italian edition of Scientific American) 1993; 229: 50-57.
15. Young R A, Elliott T J. Stress proteins, infection, and immune surveillance. *Cell* 1989; 59: 5-8.
16. Moretta A, Tambussi G, Bottino C et al. A novel surface antigen expressed by a subset of human CD3- CD16+ natural killer cells. *J Exp Med* 1990; 171: 695-714.
17. Jewett A. Cytokine and target cell-mediated regulation of the maturation and differentiation pathways of human natural killer cells. *Diss Abstr Int (B)* 1994; 55: 810.
18. Hansson M, Kiessling R. In: Herberman R B, ed. *NK Cells: Fundamental Aspects and Role in Cancer*. Amsterdam: North-Holland, 1982.

19. Semino C, Semino A, Pietra G et al. Role of major histocompatibility complex class I expression and natural killer-like T cells in the genetic control of endometriosis. *Fertil Steril* 1995; 64: 909-916.
20. Mingari MC, Vitale C, Cambiaggi A et al. Cytolytic T lymphocytes displaying natural killer (NK)-like activity: expression of NK related functional receptors for HLA class I molecules (p58 and CD94) and inhibitory effect on the TCR-mediated target cell lysis or lymphokine production. *Int Immunol* 1995; 7: 697-703.
21. Perez-Villar JJ, Melero I, Rodriguez A et al. Functional ambivalence of the Kp43 (CD94) NK cell associated surface antigen. *J Immunol* 1995; 154: 5779-5788.
22. Lebow LT, Bonavida B. Purification and characterization of cytolytic and noncytolytic human natural killer cell subsets. *Proc Natl Acad Sci USA* 1990; 87: 6063-6067.
23. Ding-E Young J, Cohn ZA. Come agiscono le cellule killer. *Le Scienze* (Italian edition of Scientific American) 1988; 235: 28-34.
24. Mayer MM. Il sistema complemento. In: *La Nuova Immunologia*. Milano: Le Scienze Spa Editore, 1992: 172-182.
25. Grossi CE, Zarcone D, Nocera A. Morfologia e fenotipo dei linfociti umani. In: *La Nuova Immunologia*. Milano: Le Scienze Spa Editore, 1992: 89-98.
26. Roger R, Breard J, Comisso M, Bohuon C, Pallardy M, Bertoglio J. CD28-mediated cytotoxicity of YT natural killer cells on B7 positive targets induces rapid necrotic death independent of granule exocytosis. *Cell Immunol* 1996; 168: 24-32.
27. Greenberg AH, Shi L, Aebersold R. The apoptosis-inducing NK cell granule protein fragmentin is a serine protease with homology to RNKP-I and CCPI/granzyme B. *FASEB J* 1992; 6: A1692.
28. Kausalya S, Hegde SP, Bright JJ, Khar A. Mechanism of antibody-dependent natural killer cell-mediated AK-5 tumor cell death. *Exp Cell Res* 1994; 212: 285-290.
29. Shi L, Kraut RP, Aebersold R, Greenberg AH. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J Exp Med* 1992; 175: 553-566.
30. Quattrone A, Capaccioli S. I geni della morte cellulare programmata. *Le Scienze* (Sci Am) 1995; 326: 42-50.



Heat shock protein 70 (Hsp70) stimulates proliferation and cytolytic activity of natural killer cells

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We previously demonstrated that lysis of tumor cells that express Hsp70, the highly stress-inducible member of the HSP70 family, on their plasma membrane is mediated by natural killer (NK) cells. Here, we studied the effects of different proteins of the HSP70 family in combination with interleukin 2 (IL-2) on the proliferation and cytotoxic activity of human NK cells *in vitro*. Proliferation of NK cells was significantly enhanced by human recombinant Hsp70 (rHsp70) and to a lesser extent by rHsp70homC, the recombinant C-terminal peptide-binding domain derived from Hsp70hom, but not by the constitutive Hsc70 or DnaK, the *Escherichia coli* analogue of human Hsp70. Even rHsp70 protein alone moderately enhances proliferation and cytolytic activity of NK cells, thus indicating that the stimulatory effect is not strictly dependent on IL-2. NK cells stimulated with rHsp70 protein also exhibit an increased secretion of interferon γ (IFN- γ). The phenotypic characterization of NK cells with specificity for Hsp70-expressing tumor cells revealed a CD16^{dim}/CD56^{bright} and increased CD57 and CD94 expression. The cytolytic activity of NK cells also was significantly reduced when a CD94-specific antibody or rHsp70 was added directly before the cytotoxicity assay, whereas other antibodies directed against CD57 and major histocompatibility complex class I molecules or Hsp70 proteins, including Hsc70 and DnaK, did not affect the NK-mediated killing. However, long-term incubation of NK cells with rHsp70 protein enhances not only the proliferative but also the cytolytic response against Hsp70-expressing tumor cells. Our results indicate that the C-terminal domain of Hsp70 protein affects not only the proliferative but also the cytolytic activity of a phenotypically distinct NK cell population with

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Introduction

The search for molecules that elicit cancer immunity led to the identification of chaperones with molecular weights of 70 and 90 kDa [1–4]. In addition to their intracellular chaperoning function for tumor peptides [5], members of the 70-kDa heat shock protein family (HSP70) elicit a strong anti-cancer immunity if they are expressed on the cell surface and thus are accessible to immunocompetent effector cells [6]. Cell surface expression of cytosolic Hsp70 on human tumor cells has been demonstrated by flow cytometry, light and electron microscopy, erythrorosetting, and/or selective cell surface protein biotinylation, although the mechanism for transport and anchorage in the plasma membrane remains to be elucidated [6–9]. Evidence is accumulating that, similar to rapidly released proteins including interleukin 1 (IL-1) or basic fibroblast growth factor [10–13], Hsp70 is transported to the plasma membrane as a protein complex via a nonclassical, endoplasmic reticulum-Golgi independent pathway [9,14,15].

In vitro, certain carcinoma cell lines, including colon carcinoma cells CX2, express Hsp70 on their plasma membrane already under physiologic conditions [8,16]. By cell separation experiments, two colon carcinoma sublines, CX+ and CX– were generated that differ with respect to their capacity to express Hsp70 on the plasma membrane but exhibit an identical major histocompatibility complex (MHC) and adhesion molecule expression pattern. Functionally, the inducible and constitutive plasma membrane

A portion of Dr. Multhoff's research was conducted in his former institution University Hospital, Med III, LMU Munich.

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Table 1. HLA antibodies and antisera used to define HLA antigens

Name of the antibody (reference)	HLA specificity	Reactivity to	
		CX+	CX–
2536 (Grosse-Wilde)	A1	–	–
880019 (Ochsner)	A2, A69, B57	+	+
HB117	A2, A69	+	+
Fe44 (Ferrara)	A2	+	+
843609 (Grosse-Wilde)	A9	–	–
GSWS (Grosse-Wilde)	A10	–	–
823258 (Grosse-Wilde)	A11	–	–
LIDDELL (Darke)	B14, B18, B39, Bw55	–	–
JB2 (Wank)	B14, B18	–	–
GIBBS (Terasaki)	B15, B22, B40	+	+
STEELE (Duquesnay)	B17	–	–
LASELL (Duquesnay)	B22	+	+
S4 (Johnson)	B44	+	+
KT4 (Johnson)	B44, B45, B51, B52	+	+
DOUGHTY (Duquesnay)	B65	–	–
HLA C alleles	HLA Cw1,2,3,4,5,6,7,8	–	–

Furthermore, HLA class I and class II alleles were defined on CX+ and CX– sublines by sequencing based typing with an ALF automated sequencer [19,20].

Both tumor sublines CX+ and CX– were typed confirmly as: HLA A2 0201, B44, B22, DQB1 0501, DRB1 0103; no HLA C alleles could be determined on neither one of the two sublines and on the original cell line CX2.

expression of Hsp70 on tumor cells correlate with increased sensitivity to lysis mediated by non-MHC restricted natural killer (NK) cells [3,16]. Antibody (Ab) blocking studies using Hsp70-specific monoclonal antibodies directed against the C-terminal substrate binding domain of Hsp70 revealed strong inhibition of NK-mediated lysis [16,17].

The present report characterizes some aspects of the specificity of Hsp70 recognition by NK cells and the associated effects on NK cell function in vitro, as assayed by adhesion, tumor cell cytotoxicity, and proliferative activity.

Materials and methods

Cell culture

Monocyte-depleted peripheral blood lymphocytes derived from healthy human volunteers were separated into nonadherent CD3⁺ T cells and cytokine-dependent, transient (12–24 hour) plastic adherent CD3[–] (CD16⁺/CD56⁺) NK cell subpopulations in a multi-step procedure after a 12-hour incubation period in IL-2-containing medium, as described elsewhere [16,18]. The cells were cultured separately in rIL-2 (100 IU; Chiron, Frankfurt, Germany) containing RPMI 1640 (Life Technologies, Eggenstein, Germany) medium for 3 to 4 days.

The autologous human colon carcinoma sublines CX+ and CX–, which differ with respect to their expression of Hsp70 at the plasma membrane but exhibit an identical MHC and adhesion molecule expression pattern [16], were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies), 6 mM L-glutamine, and antibiotics (100 IU/mL penicillin and 100

μg/mL streptomycin; Life Technologies). Exponentially growing tumor cells were used as target cells in cytotoxicity assays.

The cell lines were screened regularly and were defined as negative for mycoplasma contaminations (Genprobe; H. Biermann, Bad Nauheim, Germany).

HLA typing of human colon carcinoma sublines CX+ and CX–

The tumor sublines CX+ and CX– could not be HLA typed by serologic methods; therefore, the HLA antigens were defined by flow cytometry (FACSscan analysis, described later) using the HLA antibodies and antisera listed in Table 1.

Furthermore, HLA class I and class II alleles were defined on CX+ and CX– sublines by sequencing-based typing with an ALF automated sequencer [19,20].

Both tumor sublines CX+ and CX– were typed confirmatively as HLA A2 0201, B44, B22, DQB1 0501, DRB1 0103; no HLA C alleles could be determined on either one of the two sublines and on the original cell line CX2.

Hsp70 protein panning

The Hsp70 proteins used in this study were as follows: rHsp70 (recombinant human Hsp70A expressed in *Escherichia coli*, SPP-755), Hsc70 (constitutive form of Hsp70 purified from bovine brain, SPP-750), and DnaK (Hsp70 homolog purified from *E. coli*, SPP-630) were obtained from StressGen Biotechnologies (Victoria, Canada). Hsp70homC (aa 384–561) is the recombinant C-terminal peptide binding domain of Hsp70hom expressed in *E. coli*. All proteins were diluted in phosphate-buffered saline (PBS) at a stock concentration of 1 mg/mL and frozen in aliquots at –80°C. The sequence homologies of all proteins over the complete and over the C-terminus are compared in Figure 1.

Panning experiments were performed using recombinant human Hsp70 protein or DnaK. The proteins were diluted in PBS at a stock concentration of 1 mg/mL and frozen at –80°C. Briefly, T-25 culture flasks were incubated with Hsp70 proteins (10 μg/mL) diluted in 3 mL of ice-cold carbonate buffer at pH 9.5 for 12 hours. Following blocking of nonspecific binding sites with PBS/5% fetal calf serum (FCS), peripheral blood mononuclear cells suspended in PBS/1%FCS/0.1% sodium azide were incubated in the culture flask for 30 minutes at room temperature. Nonadherent cells were obtained from the initial supernatant fraction postincubation. Adherent cells were harvested by sequential washing steps using ice-cold PBS/10% FCS solution. To remove slightly adherent cells, a single, mild washing step was used. Strongly adherent cells were obtained by additional, stringent washing. The different cell populations obtained at each washing step were counted separately and phenotypically characterized by flow cytometry using antibodies directed against CD3 as a T-cell marker and CD16/CD56 as NK cell markers.

³H thymidine uptake

The proliferative capacity of T or NK cells against different Hsp70 proteins was determined in a standard ³H thymidine uptake assay [21]. Viable cells (5 × 10⁴ cells/100 μL) were seeded in a 96-well flat-bottom microtiter plate (Greiner, Nürtingen, Germany) in supplemented RPMI 1640 medium containing 100 IU IL-2 and different recombinant Hsp70 proteins (rHsp70, Hsc70, DnaK, rHsp70homC). By testing different concentrations of the Hsp70s (1, 10, 50, 100 μg/mL), a final concentration of 10 μg/mL proved to be optimal for stimulation. As an internal control, the proliferative activity against IL-2 (100 IU/mL) was determined in parallel.

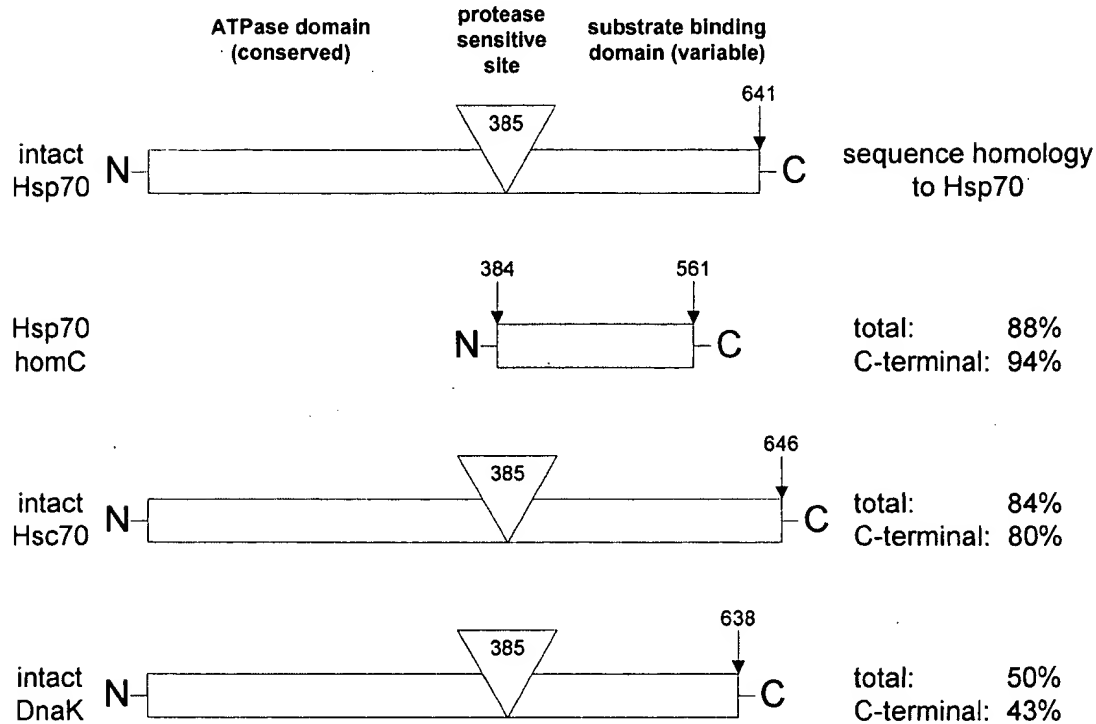


Figure 1. Sequence homology of different Hsp70 proteins. Comparison of the sequence homology of different recombinant Hsp70 proteins: rHsp70 (aa 1–641) [39], C-terminal part of rHsp70hom (aa 384–561) [40], Hsc70 (aa 1–646) [41], and DnaK (aa 1–638) [42].

After a 24- or 48-hour incubation period, the cells were pulsed with ^3H thymidine (1 $\mu\text{Ci}/\text{well}$) and the total uptake was measured following an 18-hour incubation period at 37°C in a liquid scintillation counter (Beckmann Instruments, Munich, Germany).

Monoclonal antibody, FACScan analysis, and cell sorting

The following antibodies (Ab) were used for phenotypic characterization of T and NK cells. Antibodies used for cell sorting are marked with an asterisk: isotype-matched control Ab (Dianova, Hamburg, Germany), CD3/CD16/CD56 (SimultestTM, Becton Dickinson, Heidelberg, Germany), CD45/CD14 (SimultestTM, Becton Dickinson), CD16 (Dianova), and CD3* (Dianova). The expression of killer cell inhibitory receptors (KIR) [22] was determined using monoclonal antibody 1846 (p58.2; Immunotech, Hamburg, Germany), 1847 (p58.1, Immunotech), 1614 (CD94, Immunotech), and 5.133 supernatant (p70, kindly provided by Dr. D.J. Schendel).

Flow cytometry was performed as described elsewhere [6] on a FACScan instrument (Becton Dickinson). The percentage of positively stained cells was defined as the difference between the number of specifically stained, viable (propidium iodide negative) cells minus the number of cells stained with isotype-matched control antibodies.

Monocyte-depleted peripheral blood lymphocytes were separated into a CD3⁺ T-cell population and a CD3[−] NK cell population by cell sorting on a FACStar^{plus} instrument (Becton Dickinson). The purity of each cell fraction was determined by flow cytometry.

Enzyme-linked immunosorbent assays

Tumor necrosis factor α (TNF- α) and IFN- γ in the supernatant of either untreated or Hsp70-treated NK cells incubated with different concentrations of IL-2 was determined by an enzyme-linked immunosorbent assay sandwich technique as described elsewhere [23]. Briefly, 96-well plates were coated with the antibodies against the different chemokines and cytokines and then incubated with the relevant cell culture supernatants or cytokine standards, respectively. Detection was performed with the biotinylated antibodies and a peroxidase conjugate according to the manufacturer's instructions.

Cytotoxicity and inhibition assays

NK-mediated cytotoxicity was measured using a 4-hour ^{51}Cr radioisotope assay [16,24]. Inhibition assays were performed by preincubation of NK cells with Hsp70 proteins (rHsp70, Hsc70, DnaK, rHsp70homC) at a final concentration of 5 $\mu\text{g}/\text{mL}$ (1×10^6 cells) for 30 minutes at room temperature directly before the assay. As target cells, the autologous colon carcinoma sublines CX+ and CX− were used. These sublines differ with respect to their capacity to express Hsp70 on the plasma membrane but exhibit an identical MHC and adhesion molecule expression pattern. The percentage of specific lysis was calculated as: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] \times 100. Spontaneous release in all experiments was always $<15\%$.

Inhibition assays were performed by preincubation of NK cells with antibodies directed against CD94 or CD57 (5 μg per 1×10^6 cells) and tumor target cells with antibodies directed against MHC class I antigens. Following a 30-minute incubation period at room

temperature, the NK cells were used for the cytotoxicity assays without washing as described earlier.

Results

Stimulation of T and NK cell proliferation by rHsp70

The proliferation of purified NK and T cells, derived from the same donor, stimulated with the HSP70 proteins rHsp70,

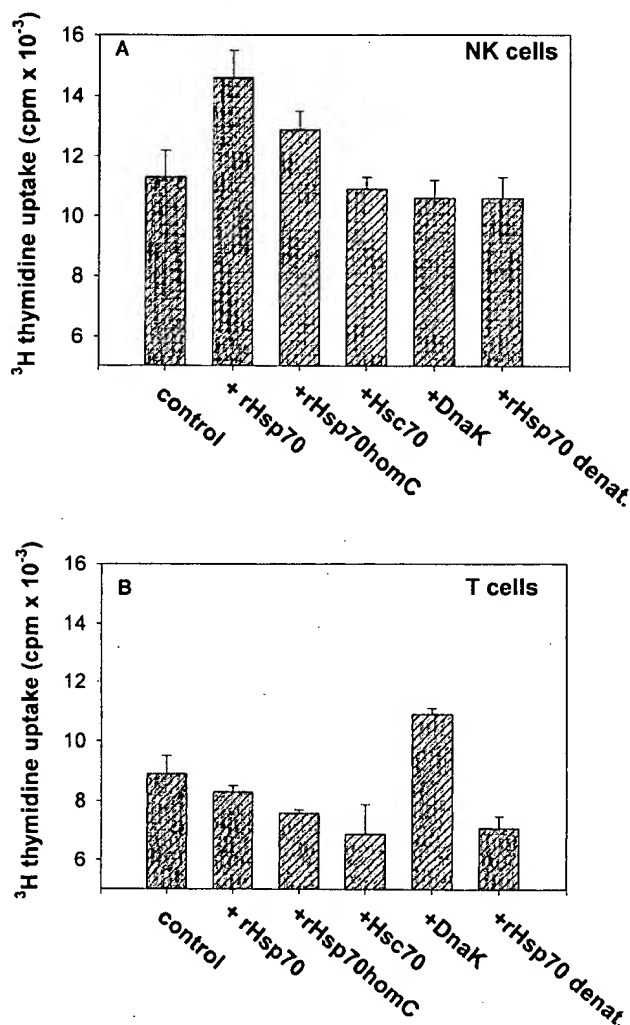


Figure 2. Stimulation of the proliferative activity of NK cells, but not T cells, by rHsp70 and rHsp70homC. Comparison of the proliferative activity of separated NK cells (A) and T cells (B) stimulated either with IL-2 alone (100 IU/mL; control) or with different recombinant Hsp70 proteins (rHsp70, rHsp70homC, DnaK, Hsc70, and heat-denatured rHsp70) diluted in IL-2 medium (100 IU/mL) at a concentration of 10 µg/mL each. Phenotypic characterization of NK cells: CD3: <5%; CD16/CD56: 46–87%; CD94: 60–70%; p58.1 and p58.2: <20%; p70: <5% and T cells: CD3: 85–92%; CD16/CD56: 5–10%; CD94: <29%; p58.1 and p58.2: not tested; p70: not tested as determined by flow cytometry. Proliferation of the cells was measured after 48 hours and an incubation period with ³H thymidine (1 µCi/mL) incorporation at 37°C for 18 hours. Values are given as the means of three to five independent experiments ± SD.

Table 2. Comparison of the proliferative activity of NK stimulated either with IL-2 at different concentrations or with IL-2 plus recombinant Hsp70 protein (rHsp70) at a concentration of 10 µg/mL each

	³ H thymidine (cpm)	
	IL-2	IL-2 + rHsp70
IL-2		
0 IU/mL	633 ± 83	900 ± 97
1 IU/mL	1277 ± 193	1602 ± 113
10 IU/mL	2956 ± 389	3987 ± 245

Proliferation of the cells was measured after 48 hours and an incubation period with ³H thymidine (1 µCi/mL) incorporation at 37°C for 18 hours. Values are given as means of three independent experiments ± SD.

DnaK, Hsc70, and rHsp70homC (aa 384–561), was measured in a standard ³H thymidine uptake assay. Dose escalation studies using different Hsp70 proteins at concentrations ranging from 1 to 100 µg/mL revealed that a maximal stimulation of the proliferative capacity could be obtained with 10 µg/mL Hsp70 protein (data not shown). The proliferative activity of isolated NK and T cells was tested following in vitro stimulation with either rHsp70, DnaK, Hsc70, or rHsp70homC (aa 384–561). A comparison of the sequence homology of these members of the HSP70 family is shown in Figure 1. As summarized in Figure 2A, NK cell proliferation was significantly stimulated by rHsp70 and to a lesser extent by rHsp70homC, which is 94% identical to Hsp70 in the C-terminal domain (aa 384–561). In contrast, DnaK and Hsc70 did not stimulate NK cell proliferation under these conditions. Heat denaturation of rHsp70 completely eradicated the stimulatory effect of Hsp70 on NK cell proliferation.

By comparison, the proliferation of CD3⁺ T lymphocytes was induced by DnaK, whereas rHsp70, Hsc70, rHsp70homC, and heat-denatured rHsp70 had no significant effect on proliferative capacity (Fig. 2B). The proliferation of NK cells could be induced by human Hsp70 equivalents (rHsp70, rHsp70homC), whereas T-cell proliferation was selectively stimulated by bacterial Hsp70 (*E. coli* DnaK). With respect to the T-cell population, stimulation with DnaK did not stimulate the expression of any of the cell surface markers tested.

To evaluate whether the stimulatory effects of rHsp70 protein on NK cells is dependent on IL-2, different concentrations of IL-2 (0, 1, and 10 IU/mL) were tested. As listed in Table 2, even rHsp70 protein alone is able to induce a moderate proliferative response in NK cells.

Cytokine secretion of NK cells following incubation with rHsp70

The cytokine secretion pattern was measured by enzyme-linked immunosorbent assay technique. As shown in Table 3, a significant increase in the secretion of IFN-γ is already

Table 3. Comparison of the cytokine secretion pattern of purified NK cells treated either with IL-2 (100 IU/mL) alone or with IL-2 plus rHsp70 (10 µg/mL) for 4 days as determined by ELISA technique

	Cytokine secretion (pg/mL)	
	IFN-γ	TNF-α
0 IU IL-2	85 ± 27	>5
1 IU IL-2	164 ± 10	>5
10 IU IL-2	174 ± 25	25 ± 4
100 IU IL-2	1782 ± 210	39 ± 6
0 IU IL-2 + rHsp70	143 ± 23	29 ± 6
1 IU IL-2 + rHsp70	727 ± 82	48 ± 7
10 IU IL-2 + rHsp70	1018 ± 117	39 ± 3
100 IU IL-2 + rHsp70	2865 ± 237	65 ± 6

Data are given as the means of two independent experiments. Significant changes as compared to initial values (treatment with IL-2 only) are indicated in bold.

observed when NK cells were incubated with low concentrations of IL-2 (10 IU/mL) plus rHsp70. However, a synergistic effect on proliferation and cytokine secretion is observed when a combined treatment of rHsp70 and IL-2 at a concentration of 100 IU/mL was used (Fig. 2A). At all concentrations of IL-2 ± rHsp70, the secretion of TNF-α appeared to be unaffected.

Characteristics of NK cells with specificity for Hsp70-expressing tumor cells

The phenotypic characterization of isolated NK cells was tested following in vitro stimulation with either IL-2 alone or with IL-2 plus rHsp70. As summarized in Table 4, transient plastic adherent NK cells exhibited CD16, CD56, CD57, and CD94 expression, but did not express CD3 and T-cell receptors. Following incubation with rHsp70, the

Table 4. Phenotypic characterization of purified NK cells (purity >90%) treated either with IL-2 (100 IU/mL) alone or with IL-2 plus rHsp70 (10 µg/mL) for 4 days as determined by flow cytometry

	Percent positively stained cells (mean fluorescence values)	
	IL-2	IL-2 + rHsp70
Isotype control	2 (10)	3 (17)
CD3 and TCR	<5 (7)	<5 (6)
CD16	46–65 (862)	66–87 (316)
CD56	56–71 (348)	76–88 (485)
CD57	44–58 (404)	68–75 (672)
p58.1	6–20 (42)	5–10 (22)
p58.2	7–15 (83)	6–13 (50)
p70	<5 (10)	<5 (14)
CD94	47–68 (135)	84–91 (237)

Data are given as the range of three independent experiments. Significant changes to initial numbers as determined by the Student's *t*-test ($p < 0.01$) in the percentage of positively stained cells and in the mean fluorescence intensity after incubation with rHsp70 protein are indicated in bold.

mean fluorescence intensity of CD16 was significantly downmodulated, whereas the expression of CD57 was strongly induced. Determination of KIR on NK cells in response to a stimulation with rHsp70 revealed a significant increase in the expression of the C-type lectin receptor CD94, whereas that of the immunoglobulin-like receptors p58.1, p58.2, and p70 was not affected. CD2 expression remained unaltered (data not shown).

Effect of Hsp70 proteins on the cytotoxic activity of NK cells

Functional analysis of NK cells using Hsp70-expressing (CX+) and Hsp70-nonexpressing (CX-) tumor cells revealed that plasma membrane expression of Hsp70 correlated with an increased sensitivity to NK-mediated lysis. This NK-mediated tumor cell lysis can be blocked by prior incubation of the tumor cell lines with monoclonal antibody directed against the C-terminal region (504–617) of Hsp70 (3A3, kindly provided by S. Fox, Northwestern University) and RPN1197 (Amersham Buchler, Braunschweig, Germany) [3,16,17]. HLA typing of the two tumor sublines CX+ and CX- clearly indicates that the two sublines differ only with respect to their capacity to express Hsp70 on their plasma membrane but exhibit an identical HLA expression pattern. As shown in the Materials and methods section, CX+ and CX- tumor cells uniformly express HLA A2 0201, B22, B44, DQB1 0501, DRB1 0103 alleles, thus indicating that both sublines are derived from the same donor cell line CX2. Here, we analyzed the effects of different recombinant Hsp70 proteins (rHsp70, rHsp70homC, DnaK, Hsc70) on the cytolytic activity of NK cells against autologous Hsp70-expressing and Hsp70-nonexpressing (CX+) and (CX-) tumor cells. A comparison of the sequence homology of different Hsp70 proteins used in this assay is shown in Figure 1.

Results from cytotoxicity assays using highly purified NK cells that had been preincubated with different Hsp70 proteins at a concentration of 5 µg/mL are summarized in Figure 3. Consistent with the ability of Hsp70-specific Ab directed against the C-terminal region to inhibit NK lysis of Hsp70-expressing tumor cells [16,17], incubation of NK cells with rHsp70 during the assay inhibited cytolytic activity against CX+ tumor cells. This treatment had no significant effect on the lysis of CX- tumor cells. Similar results were obtained using rHsp70homC that also inhibited NK lysis of CX+ tumor cells, but to a lesser extent. Hsp70homC revealed the highest sequence homology to Hsp70 within the variable C-terminal region (94% homology). In contrast, neither Hsc70 (84% homology) nor DnaK (50% homology) significantly affected NK-mediated cytotoxicity of CX+ or CX- tumor cells.

Long-term stimulation of NK cells with Hsp70 protein

We were able to demonstrate that repeated incubation of NK cells with rHsp70 protein every 4 days stimulated the proliferation of NK cells (data not shown). The cytotoxic response

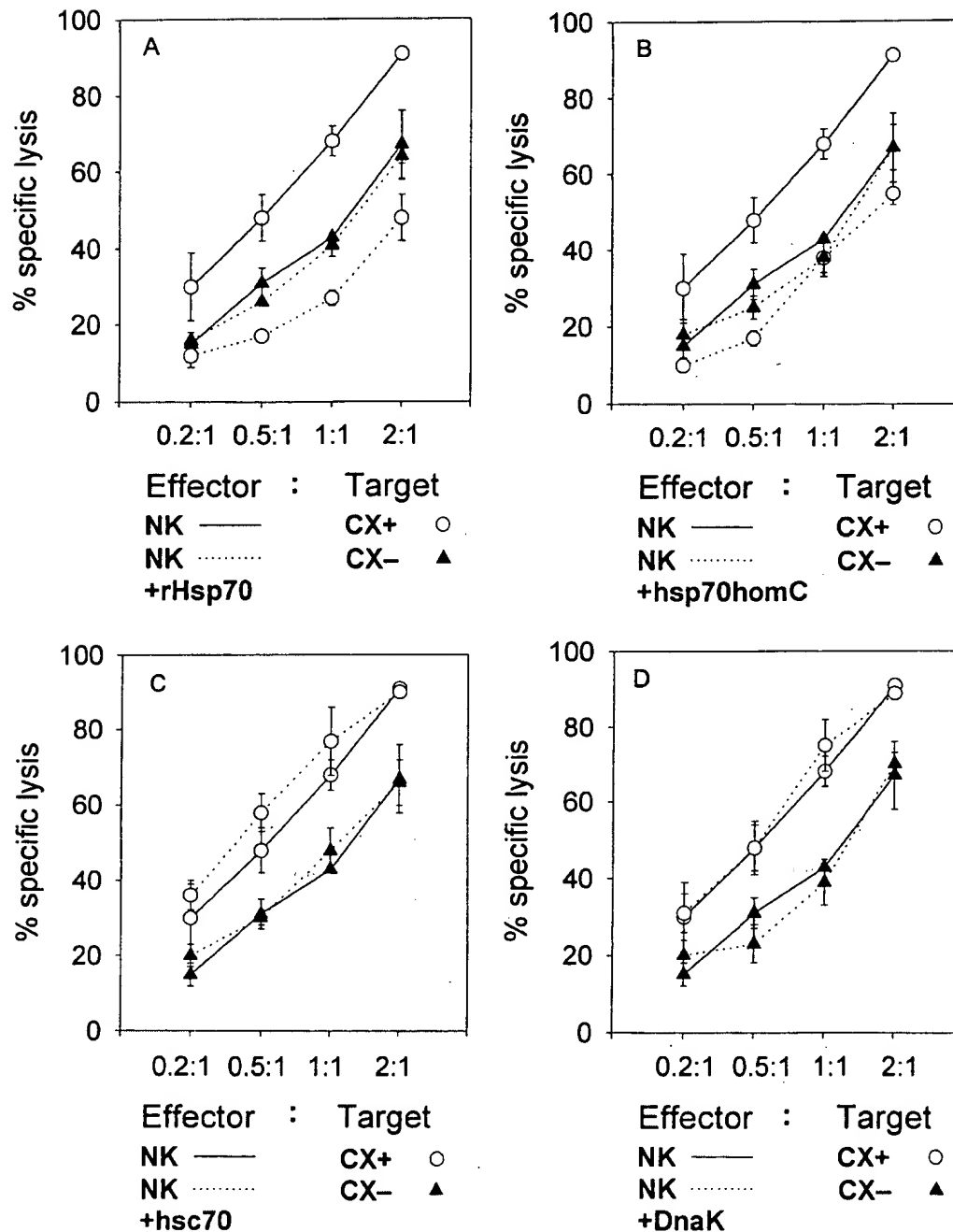


Figure 3. Blocking of the cytotoxic activity of NK cells in the presence of rHsp70 or Hsp70homC. Blocking of the cytotoxic activity of highly purified NK cells (CD3: <2%; CD16/CD56: 75–80%; CD94: 65–87%; p58.1 and p58.2: 20–30%; p70: <10%) with rHsp70 (A), rHsp70homC (B), Hsc70 (C), and DnaK (D) proteins (5 μ g/mL each; dotted lines) against 51 Cr-labeled tumor target cells, CX+ and CX–, which differ with respect to their capability to express Hsp70 on their plasma membrane. The lysis of CX+ and CX– cells mediated by NK cells that had not been incubated with Hsp70 proteins are indicated as solid lines. Results are expressed as the percentage of lysis at varying E:T ratios ranging from 0.2:1 to 2:1. Each data point represents the mean of at least three independent experiments \pm SD. The percentage spontaneous release for each tumor target cell line was always <15%.

against Hsp70-expressing CX+ tumor cells as determined on day 24 also was enhanced. NK cells that were stimulated only with IL-2 lost the cytolytic activity against Hsp70-expressing tumor cells after 24 days (Fig. 4). In general, the lytic activity of NK cells that had been stimulated only with

IL-2 compared to that of IL-2 plus rHsp70-stimulated NK cells was lower. It is important to note that the last addition of fresh rHsp70 protein has to be performed at least 4 days prior to the cytotoxicity assay to avoid the direct inhibitory effects of rHsp70 on the cytolytic activity of NK cells (see Fig. 3).

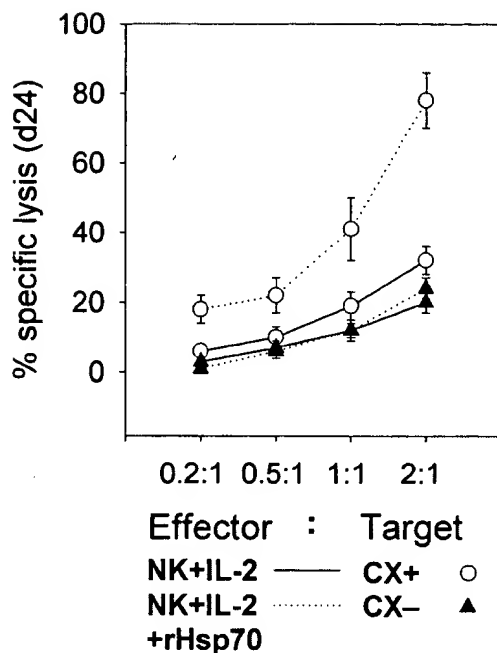


Figure 4. Long-term incubation of NK cells with rHsp70 results in increased activity against Hsp70-expressing tumor cells. Cytotoxic activity of NK cells after long-term stimulation (day 24) with IL-2 (100 IU/mL) in combination with rHsp70 protein (10 μ g/mL 1×10^6 cells) against CX+ and CX- tumor cells (dotted lines). NK cells were incubated with fresh rHsp70 protein every 4 days. The cytotoxic activity of NK cells was measured on day 24. To avoid the inhibitory effect of rHsp70 protein as shown in Figure 3, the last stimulation with fresh rHsp70 protein was performed 4 days prior to the cytotoxicity assay. The lysis of CX+ and CX- cells mediated by NK cells of the same donor that had been stimulated only with IL-2 (day 24) is indicated as solid lines. Results are expressed as the percentage of specific lysis at varying E:T ratios ranging from 0.2:1 to 2:1. The percentage spontaneous release for each target cell line was always <15%. An irrelevant isotype-matched control antibody had no inhibitory effect on the lysis of CX+ and CX- cells (data not shown).

Inhibition of lysis of

Hsp70-expressing tumor cells by CD94-specific Ab

Given our finding that the expression of the C-type lectin receptor CD94 was upregulated on NK cells following stimulation with rHsp70, one might speculate that CD94 has a dual role, acting as a KIR with specificity for certain HLA alleles and as a potential receptor for Hsp70. In support of this hypothesis, data derived from Ab blocking studies using an Ab directed against CD94 were obtained. As shown in Figure 5, a significant inhibition of lysis of Hsp70-expressing CX+ tumor cells that was mediated by rHsp70-stimulated NK cells was observed when NK cells were pre-incubated with CD94-specific Ab, whereas the lysis of CX- cells was not affected by this Ab. However, lysis of CX+ and CX- tumor cells remained unaffected when NK cells were incubated with an Ab directed against CD57, which also was markedly upregulated following incubation with rHsp70 (data not shown). In addition, the influence of

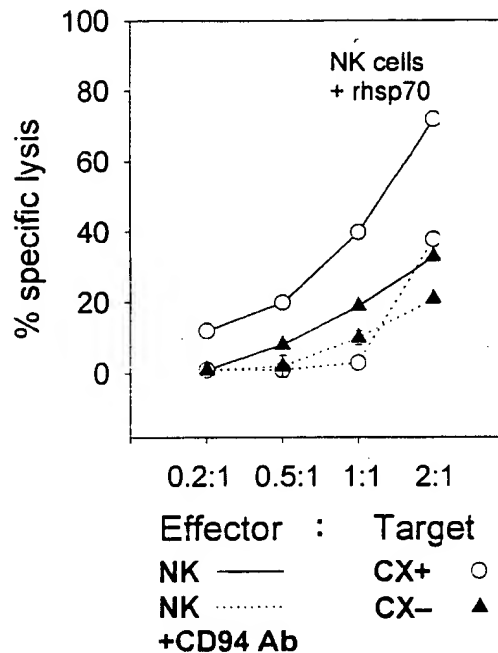


Figure 5. Blocking of the cytotoxic activity of NK cells using antibodies directed against C-type lectin receptor CD94. Antibody blocking was performed using either anti-CD94 or anti-CD57 specific Ab on NK cells at a concentration of 5 μ g/mL for 1×10^6 NK cells and MHC-specific Ab on tumor target cells. The 51 Cr release assay was performed on day 4 following the last stimulation with rHsp70 to avoid a direct inhibitory effect of rHsp70 protein. Results are expressed as the percentage of specific lysis at varying E:T ratios ranging from 0.2:1 to 2:1. The percentage spontaneous release for each target cell line was always <15%. Data represent the mean of three independent experiments. An anti-CD57 antibody and MHC class I specific antibody had no inhibitory effect on the lysis of CX+ and CX- cells (data not shown).

the MHC class I expression on the cytolytic activity of NK cells was tested. Antibodies directed against MHC class I molecules did not affect the cytolytic response of CX+ and CX- tumor cells that have been shown to express identical HLA alleles [3,16].

Discussion

NK cells belong to a lymphocyte population that is clearly distinct from T and B cells because they lack the classic T-cell receptor or surface immunoglobulin G (IgG) expression. Within the last few years, a number of KIRs have been identified on NK cells that belong to the IgG superfamily (e.g., p58, p70) [25–28] or to the type II transmembrane proteins of the C-type lectin family (e.g., CD94) [29]. According to the missing self hypothesis postulated by Ljunggren and Karre [30], the expression of HLA-A, HLA-B and HLA-C alleles protects potential target cells from lysis by NK cells. Therefore, poorly immunogenic tumors with abnormal or absent MHC class I expression should be considered as ideal targets

for NK cell recognition. It has been suggested that the cytolytic activity of NK cells is initiated and regulated by the dominance or inferiority of positive and negative signals [31]. In addition to MHC alleles that act as negative regulatory signals for NK cells, results from the present study suggest that Hsp70 also has to be considered as a target structure for NK cells.

Involvement of Hsp70 in the NK-mediated immune response against cancer cells has been shown based on its expression on the surface of these cells [4]. We previously demonstrated that enhanced cytolytic activity against Hsp70-expressing tumor cells is mediated not only by IL-2-activated but also by resting NK cells [32]. Antibodies directed against plasma membrane-bound Hsp70 efficiently inhibit the lysis of Hsp70-expressing tumor cells, whereas antibodies directed against MHC class I and II molecules or against adhesion molecules (ICAM, NCAM; data not shown) exhibited no inhibitory effect [3,16]. Here, we demonstrated that incubation of NK cells with exogenous Hsp70 during the assay efficiently inhibits the lysis of Hsp70-expressing CX+ tumor cells, whereas the lysis of HLA identical CX- tumor cells that lack expression of Hsp70 on their plasma membrane remained unaffected. Similar results were obtained with rHsp70homC, compromising the predicted C-terminal peptide-binding domain of Hsp70hom, which is 84% identical to Hsp70 over the whole sequence [33,34] and 94% identical over the peptide-binding domain 384–561. However, treatment of NK cells with other members of the HSP70 family with lower sequence identity to human inducible Hsp70, such as *E. coli* DnaK or Hsc70, did not affect the cytolytic activity against the same tumor targets.

Panning studies revealed that predominantly CD3⁻, CD16^{dim}/CD56^{bright} NK cells, but not CD3⁺ T lymphocytes adhere to rHsp70-coated substrate (data not shown). Taken together, these data strongly support the hypothesis that receptors exist on NK cells that are highly specific for the C-terminal domain of Hsp70. Following interaction with rHsp70, these receptors not only modulate the cytolytic but also the proliferative response of NK cells. Recombinant Hsp70 protein as well as the C-terminal domain of Hsp70 alone are already capable of inducing NK cell proliferation, which can be enhanced further by addition of IL-2. This finding is in line with the observation that receptors of the p58/p50 family induce both trigger of the cytolytic activity and proliferative response [22].

The secretion of IFN- γ , which is critical for an innate immune response of NK cells, is induced by addition of rHsp70. It has been shown that a combination of T-cell-derived cytokines, including IL-15, IL-12, and IL-2, induces the production of IFN- γ by NK cells. Cytokines that activate an innate immune response have been reported to limit it by the induction of apoptosis via TNF- α [35]. In contrast to cytokine-induced secretion of IFN- γ and TNF- α that results in apoptosis, rHsp70 protein alone or in combination with IL-2 does not significantly increase production of TNF- α . Long-term stimulation of NK cells using rHsp70

and low-dose IL-2 (100 IU/mL) up to 3 weeks did not exhibit any signs of apoptosis (data not shown).

Given our finding that the expression of the low-affinity receptor CD16 was downmodulated after incubation with rHsp70, it is not very likely that these NK cells kill via Ab-dependent cellular cytotoxicity. In contrast to CD16 expression, the C-type lectin receptor CD94 [36] and HNK1 (CD57) were upregulated on NK cells following stimulation with rHsp70. Therefore, one might speculate that either CD57 or CD94 is involved in the recognition of Hsp70-expressing tumor cells. The finding that Abs directed against CD94 but not against CD57 are able to inhibit the lytic activity of NK cells against Hsp70-expressing tumor cells supports the hypothesis that CD94 might be involved in the interaction of NK cells with Hsp70. That CX+ and CX- tumor cells are derived from one tumor cell line CX2 (tumor bank DKFZ Heidelberg) and exhibit an identical HLA class I expression pattern led us to the hypothesis that the recognition of Hsp70 might be mediated by a yet unknown heterodimeric CD94 lectin-type receptor that is clearly different from the HLA-specific heterodimeric receptor. The crystal structure of the extracellular domain of CD94 revealed extensive hydrophobic interfaces that facilitates dimerization with NKG2 and represents a putative ligand-binding region for HLA-E [37]. The finding that HLA class I specific antibodies did not affect the cytolytic activity of NK cells against Hsp70-expressing (CX+) and Hsp70-non-expressing (CX-) tumor cells supports the speculation that HLA-E and Hsp70 recognition has to be mediated by different CD94 receptors. Presently, studies are ongoing to identify the molecular basis of the Hsp70-specific receptor on NK cells and the biologic consequences for NK cell function.

It should be noted that other Hsp70 proteins, including DnaK and Hsc70, did not affect NK cell activity, although they were prepared and purified using procedures comparable to those used for rHsp70. Comparison of the deduced amino acid (aa) sequences of the HSP70 multigene family [33,34] indicates that the greatest divergence amongst family members is localized to the C-terminal region (aa 512–605), which contains the substrate binding domain [38]. It is noteworthy that in this particular region there is a high level of identity (94%) between Hsp70 and Hsp70homC (aa 384–561), which in the present study had similar effects on NK cell function. These data, together with the finding that Hsp70-specific Ab (3A3), directed against the C-terminal domain of Hsp70 (aa 504–617) recognizes cell surface-expressed Hsp70 proteins on tumor cells and inhibits the cytotoxic activity of NK cells [3,16], strongly suggest that the C-terminal substrate binding domain of Hsp70 is relevant for the regulation of the cytolytic function of NK cells. Deletion analysis of the C-terminal part of Hsp70 might provide further insights into the Hsp70 epitope(s) relevant for NK cell functions.

In summary, our data imply that the C-terminal part of Hsp70 protein especially acts as a regulatory signal for an

Hsp70-specific receptor on NK cells. This receptor not only controls cytolytic activity but also influences proliferative capacity.

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References

- Feldweg AM, Srivastava PK (1995) Molecular heterogeneity of tumor rejection antigen heat shock protein gp96. *Int J Cancer* 63:310
- Palladino MAJ, Srivastava PK, Oettgen HF, Deleo AB (1987) Expression of a shared tumor-specific antigen by two chemically induced BALB/c sarcomas. *Cancer Res* 47:5074
- Multhoff G, Botzler C, Wiesnet M, Eissner G, Issels R (1995) CD3-large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72-kD heat shock protein on human sarcoma cells. *Blood* 86:1374
- Multhoff G, Hightower LE (1996) Cell surface expression of heat shock proteins and the immune response. *Cell Stress Chaperones* 1:167
- Suto R, Srivastava PK (1995) A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 269:1585
- Multhoff G, Botzler C, Wiesnet M, Müller E, Meier T, Wilmanns W, Issels RD (1995) A stress-inducible 72-kDa heat-shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. *Int J Cancer* 61:272
- Ferrarini M, Heltai S, Zocchi MR, Rugari C (1992) Unusual expression and localization of heat-shock proteins in human tumor cells. *Int J Cancer* 51:613
- Botzler C, Issels R, Multhoff G (1996) Heat-shock protein 72 cell-surface expression on human lung carcinoma cells in association with an increased sensitivity to lysis mediated by adherent natural killer cells. *Cancer Immunol Immunother* 43:226
- Multhoff G (1997) Heat shock protein 72 (HSP72), a hyperthermia-inducible immunogenic determinant on leukemic K562 and Ewing's sarcoma cells. *Int J Hyperthermia* 13:39
- Mignatti P, Morimoto T, Rifkin DB, (1992) Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released via a pathway independent of the endoplasmic reticulum-Golgi complex. *J Cell Physiol* 151:81
- Rubartelli A, Cozzolino F, Talio M, Sitia R (1990) A novel secretory pathway for interleukin-1 α , a protein lacking a signal sequence. *EMBO J* 9:1503
- Florkiewicz RZ, Majack RA, Buechler RD, Florkiewicz E (1995) Quantitative export of FGF-2 occurs through an alternative, energy-dependent, non-ER/Golgi pathway. *J Cell Physiol* 162:388
- Hightower LE, Guidon PT (1989) Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. *J Cell Physiol* 138:257
- Mathew A, Bell A, Johnstone RM (1995) Hsp70 is closely associated with the transferrin receptor in exosomes from maturing reticulocytes. *Biochem J* 308:823
- Braun JEA, Scheller RH (1995) Cysteine string protein, a DnaJ family member is present on diverse secretory vesicles. *Neuropharmacology* 34:1361
- Multhoff G, Botzler C, Jennen L, Schmidt J, Ellwart J, Issels R (1997) Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells. *J Immunol* 158:4341
- Botzler C, Li GC, Issels RD, Multhoff G (1997) Definition of extracellular localized epitopes of hsp70 involved in an natural killer cell immune response. *Cell Stress Chaperones* 3:6
- Vujanovic NL, Yasumura S, Hirabayashi H, Lin WC, Watkins S, Herberman RB, Whiteside TL (1995) Antitumor activities of subsets of human IL-2-activated natural killer cells in solid tissues. *J Immunol* 154:281
- Terasaki PI, McClelland JD (1964) Microduplex assay of human serum cytotoxin. *Nature* 204:998
- Cereb N, Maye P, Lee S, Kong Y, Yang SY (1995) Locus-specific amplification of HLA class I genes from genomic DNA: locus-specific sequences in the first and third introns of HLA-A, -B, and -C alleles. *Tissue Antigens* 45:1
- Strong DM, Ahmed AA, Thurman GB, Sell KW (1973) In vitro stimulation of murine spleen cells using a microculture system and a multiple automated sample harvester. *J Immunol Methods* 2:279
- Bottino C, Sivori S, Vitale M, Cantoni C, Falco R, Pende D, Morelli L, Augugliaro R, Semenzato G, Biassoni R, Moretta L, Moretta A (1996) A novel surface molecule homologous to the p58/p50 family of receptors is selectively expressed on a subset of human natural killer cells and induces both triggering of cell functions and proliferation. *Eur J Immunol* 26:1816
- Lindner H, Holler E, Ertl B, Multhoff G, Schreglmann M, Klauke J, Schultz-Hector S, Eissner G (1997) Peripheral blood mononuclear cells induce programmed cell death in human endothelial cells and may prevent repair—role of cytokines. *Blood* 89:1931
- MacDonald HR, Engers HD, Cerottini JC, Brunner KT (1974) Generation of cytotoxic T lymphocytes in vitro. *J Exp Med* 140:718
- Pende D, Biassoni R, Cantoni C, Verdiani S, Falco M, Di Donato C, Accame L, Bottino C, Moretta A, Moretta L (1996) The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer. *J Exp Med* 184:505
- Lanier LL, Phillips JH (1996) Inhibitory MHC class I receptors on NK cells and T cells. *Immunol Today* 17:86
- Long EO, Colonna M, Lanier LL (1996) Inhibitory MHC class I receptors on NK and T cells: a standard nomenclature. *Immunol Today* 17:100
- Lanier LL (1998) Follow the leader: NK cell receptors for classical and nonclassical MHC class I. *Cell* 92(6):705
- Phillips JH, Chang CW, Mattson J, Gumperz JE, Parham P, Lanier LL (1996) CD94 and a novel associated protein (94AP) form a NK cell receptor involved in the recognition of HLA-A, HLA-B, and HLA-C allotypes. *Immunity* 5:163
- Ljunggren HG, Kärre K (1990) In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* 11:237
- Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, Conte R, Poggi A, Moretta A, Moretta L (1996) The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med* 183:645
- Botzler C, Ellwart J, Günther W, Eißner G, Multhoff G (1999) Synergistic effects of heat and ET-18-OCH₃ on membrane expression of hsp70 and lysis of leukemic K562 cells. *Exp Hematol* 27:470
- Günther E, Walter L (1994) Genetic aspects of the hsp70 multigene family in vertebrates. *Experientia* 50:987
- Tavaria M, Gabriele T, Kola I, Anderson RL (1996) A hitchhiker's guide to the human Hsp70 family. *Cell Stress Chaperones* 1:23
- Ross ME, Caligiuri MA (1997) Cytokine-induced apoptosis of human natural killer cells identifies a novel mechanism to regulate the innate immune response. *Blood* 89:910

36. Moretta A, Vitale M, Sivori S, Bottino C, Morelli L, Augugliaro R, Barbaresi M, Pende D, Ciccone E, Lopez-Botet ML, Moretta L (1994) Human NK receptor for HLA class I molecules. Evidence that the Kp43 (CD94) molecule functions as receptor for HLA-B alleles. *J Exp Med* 180:545
37. Boyington JC, Riaz AN, Coligan JE, Brooks AG, Sun PE (1999) Structure of CD94 reveals a novel C-type lectin fold: implications for the NK cell-associated CD94/NKG2 receptors. *Immunity* 10: 75
38. Leung SM, Senisterra G, Ritchie KP, Sadis SE, Lepock JR, Hightower LE (1996) Thermal activation of the bovine Hsc70 molecular chaperone at physiological temperatures: physical evidence of a molecular thermometer. *Cell Stress Chaperones* 1:78
39. Hunt C, Morimoto RI (1985) Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proc Natl Acad Sci U S A* 82:6455
40. Milner CM, Campbell RD (1990) Structure and expression of the three MHC-linked HSP70 genes. *Immunogenetics* 32:242
41. Sadis S, Raghavendra K, Hightower LE (1990) Secondary structure of the mammalian 70-kDa heat shock cognate protein analyzed by circular dichroism spectroscopy and secondary structure prediction. *Biochemistry* 29:8199
42. Bardwell JCA, Craig EA (1984) Major heat shock gene of *Drosophila* and the *Escherichia coli* inducible DnaK gene are homologous. *Proc Natl Acad Sci U S A* 81:848

NATURAL KILLER CELL CLONES CAN EFFICIENTLY PROCESS AND PRESENT PROTEIN ANTIGENS¹

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NK cell clones obtained from three different donors were tested for their ability to present soluble proteins to Ag-specific T cell clones. All NK clones were CD2⁺CD3⁺CD56⁺, whereas the expression of CD16 varied from clone to clone. The NK cell clones were able to process and present tetanus toxoid (TT) to TT-specific T cell clones in a class II HLA restricted manner. The capacity of NK cell clones to function as APC was also observed using the house dust mite allergen *Der p 1* and the *Der p 1*-derived peptide Val89-Cys117. As with EBV-transformed B cell line, NK cell clones could present the peptide 3-13 derived from the 65-kDa heat shock protein of *Mycobacterium leprae*, but they were unable to present the whole *M. leprae* Ag. Freshly isolated NK cells, IL-2-activated NK cells, and NK cell lines expanded in vitro could also process and present TT. The ability of the different NK populations to act as accessory cells correlated with their levels of class II HLA expression. These data demonstrate that NK cell clones can efficiently function as APC, however they may be restricted in the types of Ag that they can process.

Activation of CD4⁺ Th cells by soluble protein Ag requires recognition of Ag in association with class II HLA molecules on the surface of APC. Since most soluble Ag do not bind in their native form to class II HLA molecules, APC have to capture these Ag, internalize and process them into short peptides able to bind to class II HLA molecules, and then re-expose the Ag-class II HLA complexes on the cell surface (1). Several studies have been performed to investigate the mechanism by which accessory cells uptake, process, and present Ag to T cells (1, 2). Recently, it has become clear that the efficiency of Ag uptake and the levels of biosynthesis and expression of the class II HLA molecules determine to a large extent the Ag-presenting capacity of a given cell (2, 3). In addition, cytokines produced by the APC contribute to Ag-induced T cell activation (4, 5). Besides monocytes and macrophages, other cell types, such as B lymphocytes and Langerhans cells/dendritic cells, have been demon-

strated to function as APC (5, 6).

NK cells are defined as LGL³ that do not express CD3 or any of the T cell receptor chains, but they are CD56 positive (7). Almost all of the NK cells isolated from normal donors are positive for the low affinity Fc γ receptor III (CD16). However, a minority of CD56⁺ NK cells fail to express CD16 or express CD16 at low levels (8). They are operationally defined as lymphoid cells that mediate non-HLA-restricted cytotoxicity against certain tumor and virus-infected targets spontaneously and without prior sensitization (9). Furthermore, NK cells exert a variety of functions, including regulatory functions on the adaptive immune system and on hematopoiesis and natural resistance against microbial infections, which are presumably mediated through secretion of cytokines (7-10). Recently the availability of NK cell clones provided a unique opportunity to study NK cell functions using homogeneous cell preparations purged of any contaminant cells.

The aim of the present study was to define whether highly purified NK cells and cloned NK cells can function as APC in HLA-restricted Ag-specific T cell responses. Our results demonstrated, at the clonal level, that activated NK cells can present soluble protein Ag to T cell clones. Their ability to internalize and process protein Ag was observed for both TT and *Der p 1*, which is the major allergen in extracts of the house dust mite *Der pt*. These findings indicate that in addition to their capacity to mediate non-HLA-restricted lysis of tumor cells or virus infected targets, NK cells have the ability to process and present Ag to helper T cells.

MATERIALS AND METHODS

Isolation and purification of NK cells. Purification of NK cells from Ficoll-Hypaque isolated PBMC was performed using either a Percoll gradient or separation by magnetic beads. In the first procedure, PBMC were incubated for 4 h at 37°C on Petri dishes and then passed through a nylon wool column to remove monocytes and B cells. Nonadherent lymphocytes were fractionated by centrifugation on discontinuous gradients consisting of 30% and 40% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in PBS containing 10% FCS. Low buoyant density and high buoyant density lymphocytes were isolated from the interface and bottom of the Percoll gradients, respectively. The population of lymphocytes in the interface fraction was highly enriched for cells with LGL morphology. Staining of this LGL population with the Leu11 (anti-CD16) and Leu19 (anti-CD56) mAb revealed that between 30 and 50% Leu11⁺Leu19⁺ cells were present. Leu19⁺ cells were sorted with a FACStar Plus (Becton-Dickinson). The purity of these cells was greater than 99.5% upon reanalysis. Contamination of these cells with monocytes was assessed with phycoerythrin-labeled anti-CD14 (Leu-M3; Becton-Dickinson) analyzed by FACScan (Becton-Dickinson) and found to be

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³ Abbreviations used in this paper: LGL, large granular lymphocyte; TT, tetanus toxoid; EBV-LCL, EBV-transformed B cell line; *Der pt*, *Der-matophagoides pteronyssinus*; hsp, heat shock protein.

below detection levels (<0.2%).

Before cloning, NK cells were purified by separation using magnetic beads. Briefly, 5 to 10×10^6 cells were washed in PBS containing 1% BSA and 0.1% NaN_3 for 10 min at $250 \times g$ at 4°C . The pellet was then incubated for 30 min at 4°C with Leu11 and Leu19 mAb at a concentration of $1 \mu\text{g}/10^6$ cells. After two washes in PBS/ NaN_3 /BSA, the cells were incubated with biotinylated goat anti-mouse mAb (Tago, Burlingame, CA), at $1 \mu\text{g}/10^6$ cells, for 30 min at 4°C . The cells were washed twice and the pellet was incubated with streptavidin-FITC for 15 min at 4°C . After two washes in PBS, the cells were resuspended in a solution containing magnetic particles (11) diluted 1:100 in PBS/ NaN_3 /BSA. After 5 min incubation at 4°C , the cells were loaded to an iron wool column and separated with the MACS (Miltenyi, Sunnyvale, CA), as described previously (11). The cells stained with Leu11 and Leu19 mAb were labeled with FITC avidin and were sorted to a purity of 99.5%.

Establishment and culture of NK cell clones. CD16⁺CD56⁺ cells were resuspended in Yssel's medium (12) containing 1% human serum and cloned by limiting dilution at a concentration of one to five cells/well in 96-well round bottomed plates (Titertek; Flow Laboratories, McLean, VA) in the presence of a feeder cell mixture consisting of 5×10^5 irradiated (4000 rad) allogeneic PBMC/ml, 5×10^4 irradiated (5000 rad) cells/ml of the allogeneic EBV-LCL JY, and $0.1 \mu\text{g}/\text{ml}$ purified PHA (Wellcome Diagnostics, Beckenham, United Kingdom). After 7 days, $100 \mu\text{l}$ of medium containing $20 \text{ IU}/\text{ml}$ rIL-2, kindly provided by Dr. R. Kastelein (DNAX Research Institute, Palo Alto, CA), was added to each well. After 12 to 14 days, proliferating cultures were transferred to 24-well tissue culture plates (Linbro; Flow Laboratories) and restimulated with the feeder cell mixture. At day 5, the NK clones were washed and further expanded in medium containing rIL-2 ($20 \text{ U}/\text{ml}$). At day 8 or 9, they were restimulated with the feeder cell mixture. Six to 8 days after the last stimulation, the clones were screened in functional assays.

Cytotoxic assays. Cytotoxic activity was determined using a ^{51}Cr -release assay. Effector cells were mixed with 2×10^5 ^{51}Cr -labeled target cells in $200 \mu\text{l}$ Iscove medium (Gibco, Glasgow, United Kingdom) with 0.25% BSA in U-shaped wells of a microtiter plate, spun down at $50 \times g$, and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 . The supernatants were harvested using a Skatron supernatant collection system (Skatron, Lier, Norway) and were counted in a γ -counter. The maximum release was determined after incubation of the cells in 1% Triton X-100 and the spontaneous release by measuring the release of target cells in medium only. The percentage of specific ^{51}Cr release was determined as follows:

$$\% \text{Cr release} = 100 \times \frac{[\text{experimental release} - \text{spontaneous release}]}{[\text{maximum release} - \text{spontaneous release}]}$$

The data are presented as the mean of triplicate measurements.

Preparation of APC. The NK clones used as APC were taken 6 to 8 days after they had been activated by feeder cells, irradiated at 4000 rad, and washed three times. Before they were added to the culture, the NK cell clones were labeled with anti-CD14 mAb Leu M3 to look for possible contamination by residual feeder cells. No detectable CD14⁺ cells were observed in these preparations. The EBV-LCL and fresh PBMC, used as APC, were irradiated 5000 and 4000 rad, respectively, and washed twice.

To test the APC function of freshly isolated NK cells, three sources of irradiated NK cells were used: purified NK cells directly isolated from PBMC as described above, purified NK cells cultured in vitro for 5 days in the presence of $200 \text{ IU}/\text{ml}$ of IL-2, and purified NK cell lines tested at day 5 after activation with the feeder cell mixture described above. These three different types of NK cells were preincubated overnight in the absence or in the presence of $10 \mu\text{g}/\text{ml}$ of TT in 5% CO_2 at 37°C . NK cells preincubated in medium only and Ag-pulsed cells were then irradiated, washed three times, resuspended in medium, and used as APC for the proliferative responses of TT specific T cell clones. For chloroquine treatment, the APC were suspended at $10^6/\text{ml}$ in Yssel's medium, and freshly prepared chloroquine (Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 0.1 mM, 30 min before the addition of TT at a concentration of $5 \mu\text{g}/\text{ml}$. After 5 h, the cells were washed three times with PBS and fixed with 0.05% glutaraldehyde for 1 min at room temperature. The reaction was stopped with 0.2 M lysine in PBS. The cells were washed three times and resuspended in medium.

Cloned Ag-specific T lymphocytes. The TT-specific T cell clones 827 and SP-F1, SP-F9, SP-F11, SP-F15, SP-F17, SP-F3, and SP-F14 have been obtained from the peripheral blood of a normal donor and a severe combined immunodeficient patient reconstituted with fetal liver and thymus transplantation, respectively, as described previously (12). The proliferative response of T cell clone 827 is restricted by HLA-DR3. Clones SP-F1 and SP-F11 are restricted by HLA-DR4, whereas clones SP-F9, SP-F15, and SP-F17 recognize TT in the context of HLA-DRw11. Clones SP-F3 and SP-F14 are promiscuous and recognize Ag processed and presented by any EBV-LCL, regard-

TABLE I
Phenotype and cytotoxic activity of NK clones

NK Clone	Phenotype ^a	Target Cells (% Cr release ^b)			
		K562	Jurkat	Daudi	F17
MK36	CD16 ^{bright}	65	38	22	4
MK42	CD16 ^{dim}	50	40	25	2
MK81	CD16 ⁻	10	7	3	0
LR111	CD16 ^{dim}	40	33	30	3
LR115	CD16 ^{bright}	48	36	29	4
NPK1	CD16 ^{bright}	53	48	45	1

^a All the clones were CD2⁺CD3⁺CD56⁺.

^b E:T cell ratio = 1:1.

less of its HLA phenotype (12). The cloned T cell lines, NP-12 and NP-14, specific for group I allergens of *Der pt* were generated from the blood of a patient allergic to *Der pt* by stimulating his PBMC with $1 \mu\text{g}/\text{ml}$ of a lyophilized extract of semi-purified *Der pt*⁴ (Diephuis Laboratories, Groningen, The Netherlands). These clones react with the recombinant *Der p* 1 molecule and with the *Der p* 1-derived peptide Val89-Cys117 (VGESYRYVAREGSCRRPNAQR-FGISNYC). The T cell clone, RP1511, specific for *Mycobacterium leprae*, was obtained from a patient suffering from tuberculous leprosy, as described elsewhere (13). This clone recognizes a peptide sequence on the N terminus of the 65-kDa hsp (amino acids 3 to 13; KTIAV-DEEARR), which is an immunodominant epitope for HLA-DR3-restricted, *M. leprae* hsp 65-reactive cells.

Ag-induced T cell proliferation. Nine to 12 days after the last stimulation with the feeder cells mixture, the cloned T cells were washed three times, and 2×10^4 of these cells were incubated with 2×10^4 irradiated (5000 rad) APC in the presence or absence of soluble TT at a final dilution of $5 \mu\text{g}/\text{ml}$; the *Der pt* Ag or the *Der p* 1-derived peptide Val89-Cys117 at a concentration of $1 \mu\text{g}/\text{ml}$; the *M. leprae* or the *M. leprae* hsp 65-derived peptide aa. 3 to 13, in a final volume of $200 \mu\text{l}$ Yssel's medium with 1% human AB⁺ serum. After 3 days of incubation, $1 \mu\text{Ci}$ [^3H]TdR (New England Nuclear, Dreieich, Federal Republic of Germany) was added to each well. Four hours later, the cells were harvested onto glass fiber strips using a semi-automated cell harvester, and the amount of incorporated [^3H]TdR was assessed by liquid scintillation counting. The results are expressed as the mean of triplicate cultures \pm SD. The effect of mAb on the proliferative capacity of T cell clones was determined by adding varying amounts of mAb at the onset of the cultures. The following mAb were used as ascites fluid: W6/32, which detects a common determinant on class I HLA molecules (Sera Lab, Crowley Down, United Kingdom); mAb SPV-L3, which reacts with a monomorphic determinant on HLA-DQ molecules (14); and mAb Q5/13, which detects a determinant common to HLA-DR and HLA-DP molecules (a kind gift from Dr. S. Ferrone, Medical College, Valhalla, NY) (15).

Fluorescence analysis. One hundred thousand cells were added per well of a V-bottomed microtiter plate and washed once with PBS containing 0.02 mM NaN_3 and 1% BSA. The cells were then incubated with the following mAb: the anti-CD2 mAb CLB-11 (kindly provided by Dr. R. Van Lier, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, The Netherlands), the anti-CD3 mAb Leu4, the anti-CD56 mAb Leu19, the anti-CD16 mAb Leu11 (Becton-Dickinson), and the anti-HLA-DR, DP mAb Q5/13. After 30 min at 4°C , the cells were washed twice in PBS/ NaN_3 /BSA and incubated with a 1:40 dilution of FITC-labeled F(ab')₂ fragments of goat anti-mouse IgG (Tago, Burlingame, CA) for 30 min at 4°C . After three washes, the cells were analyzed using a FACScan (Becton-Dickinson). As a monocyte marker, the anti-CD14 mAb (Leu-M3, Becton-Dickinson), directly labeled with phycoerythrin, was used.

RESULTS

Phenotype and cytotoxic activity of NK cell clones. Cloned NK cell lines were isolated from three different donors. Their phenotypic characterization and cytolytic activity are shown in Table I. All NK clones were CD2⁺CD3⁺CD56⁺ but differed in their CD16 expression. The NK clones MK36, LR115, and NPK1 were CD16^{bright}, clones MK42 and LR111 were CD16^{dim}, whereas clone MK81 did not express detectable levels of CD16. All NK

⁴ Yssel, H., H. Gascan, P. Schneider, H. Spits and J. E. de Vries. 1991. Excessive IL-4 production by allergen specific T cell clones from atopic patients is responsible for induction of IgE synthesis. Submitted for publication.

Figure 1. Proliferative responses of TT-specific CD4⁺ T cell clones SP-F1 and SP-F11, which are restricted by HLA-DR4; SP-F15 and SP-F17, which are restricted by HLA-DRw11; and 827, which is restricted by HLA-DR3, to TT (5 μ g/ml) presented either by the NK clones MK36, MK42, MK81, LR115, LR111, and NPK1 or by the autologous EBV-LCL SPS and HSY.

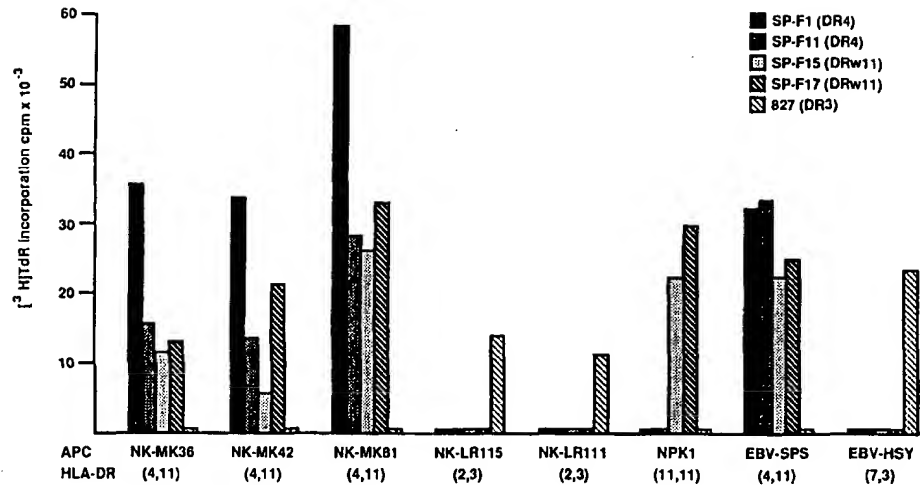


TABLE II

Effect of different Ag concentrations presented by NK cell clones on the proliferative responses of TT-specific T cell clones

Responder T Cell Clones	[TT] (μ g/ml)	APC ^a ([³ H]TdT incorporation cpm $\times 10^{-3}$)				
		Medium	MK36 ^b	MK42 ^b	MK81 ^b	SPS ^b
F1	15	2.6	40.6	38.3	42.6	58.0
	3	0.5	33.2	34.1	35.0	61.0
	0.6	0.2	15.1	17.4	18.2	55.3
	0.12	0.3	2.9	3.4	4.4	44.4
	0.024	0.1	1.9	1.5	1.9	20.7
	0.0048	0.1	0.7	1.1	1.0	0.6
	Medium	0.1	0.6	1.3	0.4	0.2
F14	15	1.3	8.3	8.2	11.3	4.4
	3	2.9	10.0	9.0	14.6	12.6
	0.6	3.8	10.4	10.1	16.0	20.8
	0.12	1.4	11.5	11.3	20.5	26.6
	0.024	0.1	4.8	5.3	15.0	22.9
	0.0048	0.1	1.0	1.2	2.2	4.8
	Medium	0.1	0.4	0.2	0.2	0.2

^a APC:responder ratio = 1:1.

^b The NK cell clones MK36, MK42, and MK81, and the EBV-LCL SPS were isolated from the same donor and are HLA-DR4,w11.

TABLE III

Effect of anti-HLA mAb on the proliferative response of T cell clone SP-F17 to TT^a

mAb	mAb ^b dilution	APC (% inhibition of proliferation)		
		SPS ^c	MK81 ^d	MK42 ^d
W6/32 (anti-HLA-A,B,C)	1:200	4	15	5
	1:600	0	12	0
	1:1800	0	15	0
SPV-L3 (anti-HLA-DQ)	1:200	2	16	1
	1:600	0	0	0
	1:1800	0	0	0
Q5/13 (anti-HLA-DP,DR)	1:200	80	75	81
	1:600	68	70	57
	1:1800	45	48	31

^a The proliferative response of F17 to TT (5 μ g/ml) in the absence of mAb was 28.9 ± 2.1 with SPS, 11.6 ± 1.0 with MK81, and 34.5 ± 2.7 with MK42 as APC (cpm $\times 10^{-3}$).

^b mAb were used as ascites fluid.

^c SPS is an EBV cell line.

^d MK81 and MK42 are NK clones originated from the same donor as SPS.

clones expressed high levels of class II HLA Ag as shown for clone MK36 (see Figure 4F). All the NK clones were highly cytotoxic for the NK-sensitive target cells K562, Daudi, and Jurkat at E:T ratios of 1:1 (Table I) or 10:1 (data not shown), except for MK81, which only killed these NK-sensitive target cells efficiently at E:T ratios of 10:1 (data not shown). On the other hand, all NK clones had no or very low cytotoxic activity against CD4⁺ TT-

TABLE IV

Ag processing by NK cell clones

APC	Treatment before Fixation	Responder T Cell Clones ([³ H]TdT incorporation cpm $\times 10^{-3a}$)			
		F3	F11	F17	F9
Medium	Medium	0.3	0.1	0.2	0.1
	Medium	0.1	0.1	0.2	0.1
MK36 (TT) ^c	TT ^b	19.8	2.0	15.0	8.9
	TT + chloroquine	9.8	0.5	0.7	0.1
	Chloroquine	32.6	19.7	17.6	ND
	Medium	ND	0.4	0.8	ND
MK81	TT	ND	18.9	22.3	ND
	TT + chloroquine	ND	1.0	4.6	ND

^a In all cases, the SD was <10% of the total cpm.

^b TT: 5 μ g/ml.

^c MK36 preincubated overnight with TT, washed, and then put in the presence of chloroquine.

specific T cell clone F17, which was included as a control, non-NK-sensitive, target cell.

NK cell clones can present TT to CD4⁺ T cell clones. To determine whether NK cell clones could process and present Ag, NK clones obtained from three different donors were tested for their capacity to present TT to CD4⁺ TT-specific T cell clones. In Figure 1, it is shown that cloned NK cells can present TT to the T cell clones SP-F1, SP-F11, SP-F15, SP-F17, and 827 in a class II HLA-restricted fashion. The cloned TT-specific T cell line SP-F1 and SP-F11 are restricted by HLA-DR4, whereas the T cell clones SP-F15 and SP-F17 recognize the Ag in the context of HLA-DRw11. All four of these clones proliferated in response to TT, presented by the DR4,w11-positive NK clones MK36, MK42, and MK81. Furthermore, the HLA-DRw11-restricted T cell clones SP-F15 and SP-F17 proliferated also in response to TT presented by the NK cell clone NPK1, which is homozygous for HLA-DRw11. The HLA-DR3-restricted T cell clone 827 recognized TT presented by the NK clones LR115 and LR111 that express HLA-DR2,3. The proliferative responses of the CD4⁺ T cell clones to optimal concentrations of TT (5 μ g/ml) presented by the NK clones were in the same range as those obtained when the class II HLA-matched EBV-LCL, SPS and HSY (Fig. 1), or PBMC (data not shown) were used as APC. This was also the case when the APC, either NK cells or the EBV-LCL, were used at suboptimal concentrations (less than 1:1 ratio; data not shown). However, at suboptimal concentrations of Ag, EBV-LCL were more efficient APC than the NK clones (Table II). At

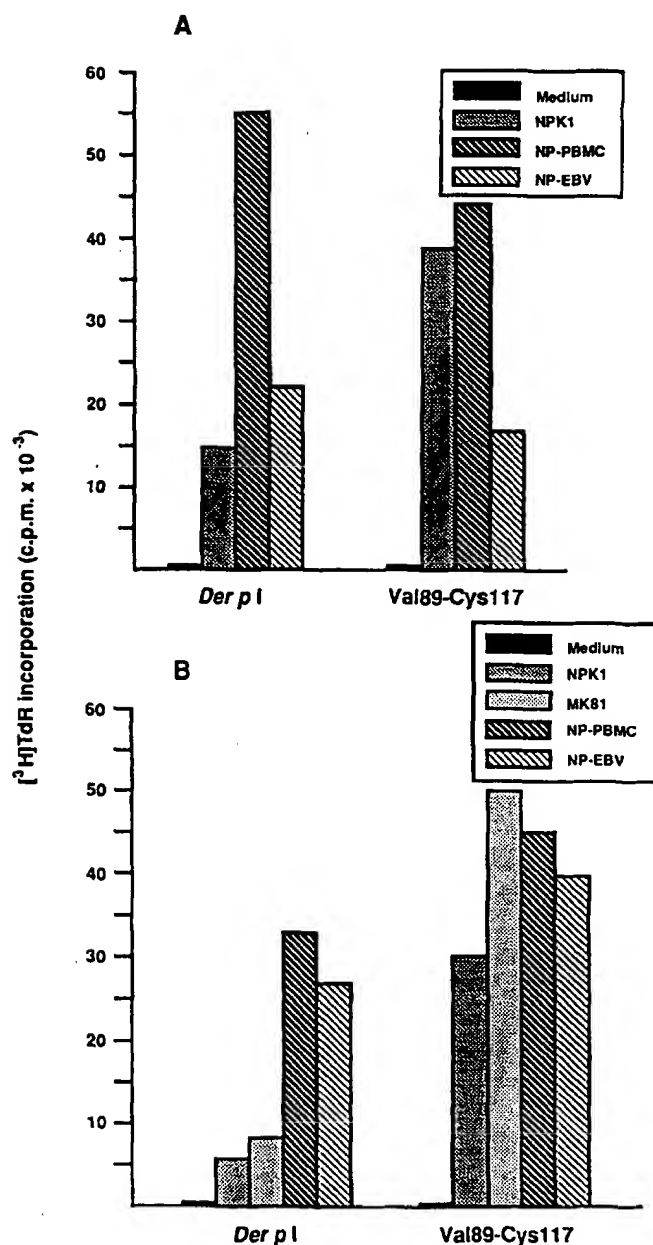


Figure 2. Proliferative responses of house dust mite-specific T cell clones NP-12 (A) and NP-44 (B) to *Der p I* Ag and to *Der p I*-derived peptide Val89-Cys117. As APC, the autologous NK cell clone NPK1, HLA-DRw11 matched NK cell clone MK81, autologous PBMC, and EBV-LCL were used.

concentrations of TT as low as 0.02 $\mu\text{g/ml}$, EBV-LCL were still able to induce moderate proliferative responses, whereas those induced by the NK cell clones were in general very low.

The notion that the TT-specific proliferative responses of the T cell clones were restricted by the HLA-DR Ag of the NK cell clones used as APC was confirmed by blocking studies with mAb against class II HLA Ag. In Table III, it is shown that the proliferative responses of T cell clone SP-F17 to TT, presented by the NK clones MK81 and MK42, were blocked by the mAb Q5/13, which is specific for HLA-DP,DR, but not by the mAb SPV-L3 and W6/32, which are directed against the HLA-DQ and the HLA class I Ag, respectively. Comparable results were found with the other TT-specific T cell clones (data not shown).

To exclude the possibility that the T cell clones recognize TT peptides, which could be present as contami-

nants in the TT preparation, the NK clones MK81 and MK36 were pulsed with TT in the presence or absence of chloroquine, which inhibits intracellular Ag processing (16). The cells were then fixed with glutaraldehyde to prevent any further processing and used as APC. As additional control, NK clone MK36 was incubated overnight with TT, washed, and then cultured in the presence of chloroquine plus responder T cells. In this case, chloroquine should not have any inhibitory effect because it was added to the culture after the uptake and processing of the Ag by the NK-APC. In Table IV, it is shown that the NK cell clones pulsed with TT and then fixed with glutaraldehyde can present Ag to the T cell clones SP-F3, SP-F11, SP-F17, and SP-F9. The addition of chloroquine during the Ag pulse resulted in strong reduction of proliferation of all four T cell clones tested. Treatment of MK36 cells with chloroquine, after the cells had been preincubated overnight with TT but before fixation, did not affect the proliferative responses of the T cell clone, indicating that chloroquine had no toxic effects. These data demonstrate that NK cell clones can internalize and process soluble protein Ag such as TT and express it on the membrane in form of a short peptide.

NK cell clones present the major house dust mite allergen *Der p I* to *Der p I*-specific T cell clones. The capacity of NK clones to present soluble proteins to Ag-specific T cell clones is not restricted to TT. In Figure 2, it is shown that the NK cell clone MK81, which is HLA-DRw11-compatible with donor NP, and the autologous NK clone NPK1, effectively present recombinant *Der p I* to the *Der p I* specific CD4⁺ T cell clones NP-12 and NP-44. In addition, NP-12 and NP-44 proliferated in response to the *Der p I*-derived peptide Val89-Cys117 presented by the NK clones. The levels of proliferation towards *Der p I* or Val89-Cys117 peptide presented by the NK clones were lower or comparable, respectively, to those obtained when the autologous EBV-transformed B cell line NP-EBV or PBMC of donor NP were used as APC.

NK cell clones fail to present whole *M. leprae*, but can present effectively the *M. leprae* hsp 65-derived peptide aa. 3-13. NK cell clones LR111 and LR115 were very inefficient in presenting the *M. leprae* to Ag-specific CD4⁺ T cell clones as compared with PBMC (Fig. 3A). This failure is not unique for NK cells, since the autologous EBV-transformed B cell lines were also inefficient APC for *M. leprae*. However, when very high concentrations (100 $\mu\text{g/ml}$) of Ag were used, low responses could be induced even using NK clones or EBV-LCL as APC. In contrast, PBMC were effective in presenting the whole *M. leprae* Ag to the specific CD4⁺ T cells. The T cell proliferation was dependent on the Ag concentrations. It has been reported previously that the monocytes present in the PBMC are responsible for uptake, processing, and presentation of *M. leprae* (17). The defective presentation of *M. leprae* by both NK cell clones and EBV-LCL is not due to their inability to present the relevant antigenic peptide, because both cell types could effectively present the 65-kDa-derived peptide 3-13 to the *M. leprae*-specific T cell clones (Fig. 3B). It is therefore likely that NK cell clones and EBV-LCL, in contrast to monocytes, are unable to uptake or process the whole *M. leprae*. However, this failure is related to the characteristic of the Ag and is not due to an intrinsic inability of the NK clones to uptake or process soluble protein Ag, since they could process TT and *Der p I* (Figs. 1 and 2).

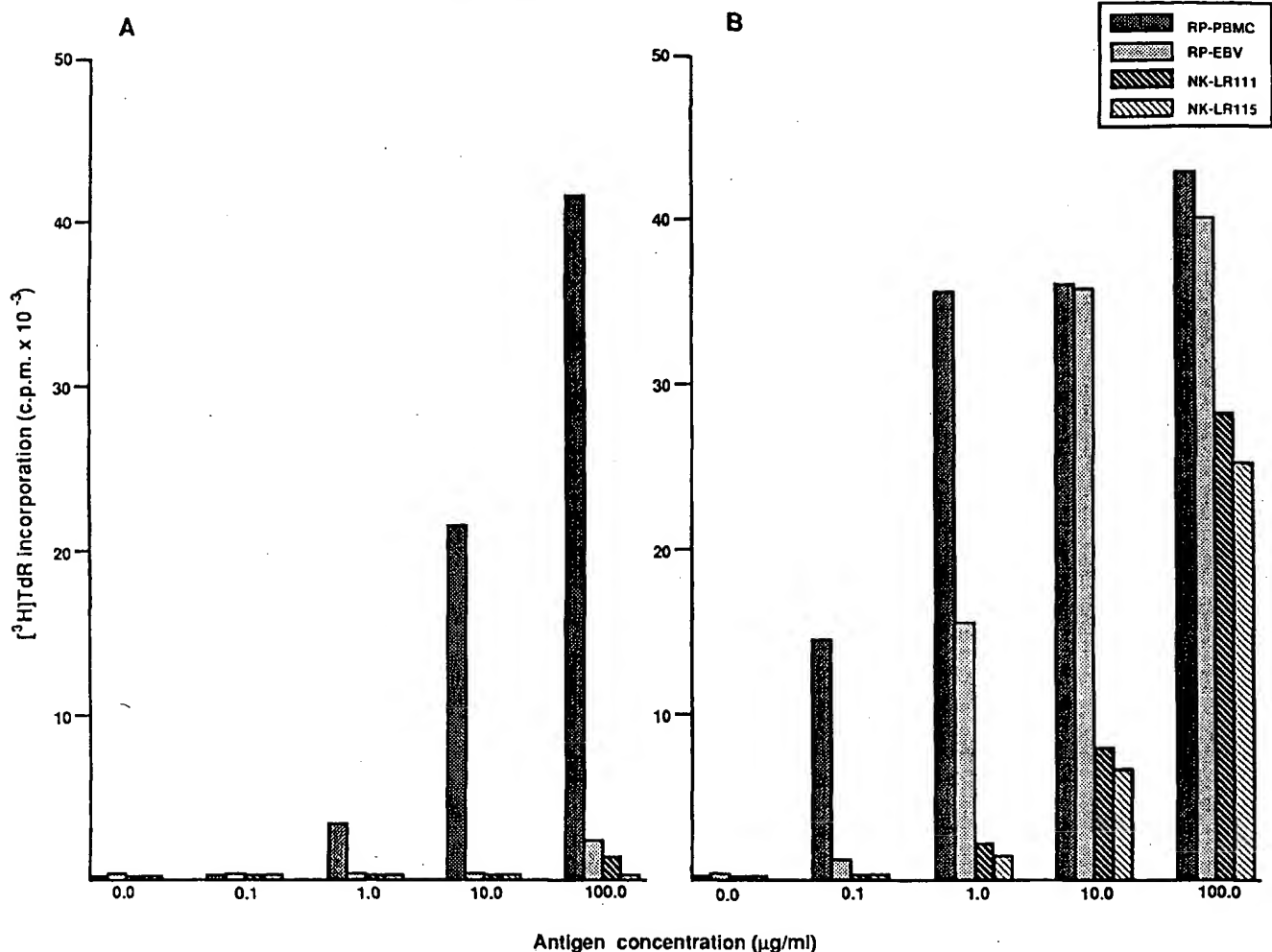


Figure 3. Proliferative responses of *M. leprae*-specific T cell clone RP-1511 to different concentrations of *M. leprae* (A) and *M. leprae*-derived peptide aa. 3-13 (B). As APC, the autologous NK clones NK-LR111 and NK-LR115, PBMC, and EBV-LCL were used.

TABLE V
Proliferative response of T cell clones to TT presented by fresh NK cells

APC ^a		Responder T Cell Clones ([³ H]TdR incorporation cpm x 10 ⁻³)	
		F3	F14
SPS	1:1 ^b	36.7 ^c	18.9
	3:1	52.1	31.3
PBMC	1:1	27.8	11.2
	3:1	40.4	17.0
NK fresh	1:1	10.0	1.2
	3:1	16.5	5.7
NK + IL-2	1:1	38.0	18.1
	3:1	29.8	20.1
NK + feeder	1:1	52.8	37.2
	3:1	53.3	38.7
Medium		1.3	0.5

^a Preincubated overnight with 10 μg/ml of TT. The proliferative responses of the T cell clones in the presence of non-Ag pulsed APC were, in every situation, comparable or less than those observed when these T cell clones were cultured in medium only (i.e., in the absence of APC).

^b APC:responder ratio.

^c Results are expressed as the mean cpm obtained in three different experiments performed on three different normal donors. In all cases, the SD was <10% of the total cpm.

APC function of freshly isolated NK cells. To investigate whether fresh NK cells can also process and present Ag, we isolated NK cells from peripheral blood of normal donors and tested their ability to present TT to the CD4⁺ T cell clones SP-F3 and SP-F14. These TT-specific clones

were used as responder cells because they have the particular property to recognize processed TT in association with any HLA-DR molecule (12). Therefore, their proliferative responses to TT can be tested using various HLA mismatched PBMC, EBV-LCL, or NK clones as APC.

As shown in Table V, fresh NK cells incubated overnight with TT have the capacity to process and present Ag. Their APC function is, however, less efficient than that of EBV-LCL or PBMC and varied from donor to donor (data not shown). The capacity to process and present TT was strongly enhanced when the NK cells were activated for 5 days with optimal concentrations of rIL-2 (200 U/ml) or with a mixture of feeder cells (as described in *Materials and Methods*). Proliferative responses of F3 and F14 were comparable when the EBV-LCL SPS, PBMC, or IL-2-activated NK cells, isolated from the same donor, were used. NK cell lines activated with feeder cells induced slightly higher T cell responses to TT. As shown in Figure 4, the different degrees of efficiency in Ag processing and presentation by freshly isolated NK, NK activated in vitro with IL-2, and NK cell lines, correlated with the different degrees of HLA class II expression by these cells.

DISCUSSION

NK cells are cytotoxic for virally infected cells and certain cancer cells and have therefore been implicated

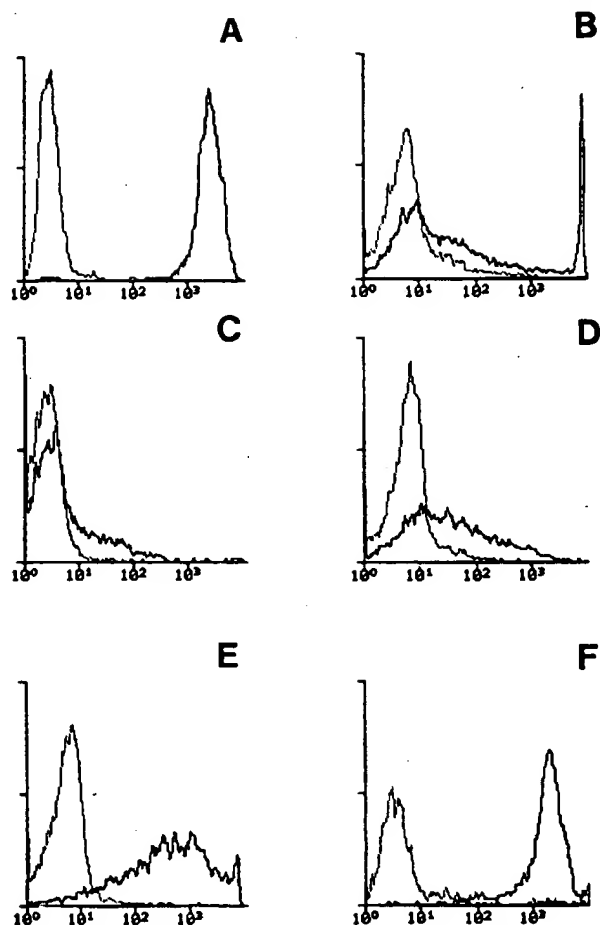


Figure 4. HLA-DR,DP expression on EBV-LCL (A), PBMC (B), freshly isolated NK cells (C), IL-2 activated NK cells (D), NK cell lines expanded with feeder mixture (E), and NK cell clone MK36 (F) was determined by FACSscan analysis using the Q5/13 mAb.

to play a major role in first line immunodefense against malignancies and viral infections (7-9). In addition, NK cells have been reported to act as natural suppressor cells in graft-vs-host reactions after bone marrow transplantation (18). Furthermore, NK cells have important immunoregulatory functions that are mediated via cytokines produced by these cells (7-10).

In the present study, we demonstrate that highly purified activated NK cells and cloned NK process and present soluble Ag to CD4⁺ Ag-specific T cell clones. The ability of NK cell clones to present Ag was not due to contaminating monocytes or EBV-LCL from the feeder cell mixture, since we did not observe detectable CD14⁺ cells in our preparations. More importantly, Ag presentation was consistently restricted by the HLA-DR of the particular NK clone. In experiments like those presented in Figure 1, several clones with different HLA-DR phenotypes cultured with the same feeder cell mixture could only present Ag to those Ag-specific T cell clones restricted by the HLA-DR of the NK clones.

In addition, our data strongly suggest that freshly isolated NK cells can also present Ag. In this case it is, however, not possible to exclude that some monocytes were still present in the purified preparation, although our NK cells were rigorously depleted by a combination of adherence, nylon wool depletion, and sorting, and we could not detect any CD14⁺ cells in these NK populations. We show that activated NK cells are generally as effective

as APC as EBV-LCL and PBMC for both TT and the major allergen for house dust mite *Der p 1*. In addition, cloned NK cells were able to present a *Der p 1*-derived 29-mer peptide. The proliferative responses of TT and *Der p 1*-specific T cell clones towards these Ag presented by cloned NK cells were restricted by HLA-DR Ag and not by HLA-DQ Ag. NK cell clones pretreated with cloroquine failed to induce productive T cell proliferation in response to TT, indicating that TT presentation required processing of the Ag and was not mediated via TT-derived peptides, which could have been present in the TT preparations, accommodated in the HLA-DR molecules. Taken together, these data indicate that activated NK cells can efficiently process and present Ag in a HLA class II-restricted fashion to T cell clones and that in this aspect they are not different from other APC such as monocytes, macrophages, or EBV-LCL. However, it remains to be determined whether NK cells can activate resting T cells, as has been shown for macrophages and dendritic cells (1, 2). Interestingly, cloned NK cells, like EBV-LCL, were very inefficient in presenting the *M. leprae*, whereas monocytes have been shown to process and present this Ag efficiently (17). Nevertheless, both the cloned NK cells and the EBV-LCL were able to present the hsp 65-derived peptide aa. 3-13. Therefore, it may be concluded that the failure of NK cells or EBV-LCL to effectively present the *M. leprae* to *M. leprae*-specific CD4⁺ T cell clones is not due to a lack of binding of the immunodominant peptide to class II HLA molecules, but is probably related to the inability of these cells to appropriately uptake or process this Ag. Similar data have been described for Ag presentation of soluble bacterial streptolysin O by purified LGL isolated by cell sorter and completely depleted of both monocytes and dendritic cells (19). However, Scala et al. (20) reported that a subset of LGL can function as accessory cells for soluble streptolysin O. Individual T cell epitopes can have processing requirements that differ greatly, therefore it is possible that NK cells and EBV-LCL can modify and process only certain types of Ag.

It has been reported that biosynthesis and expression of class II HLA molecules are crucial factors to determine the Ag presenting capacity of a given cell (3, 21). Cloned NK cells express high levels of class II HLA Ag, which may account for their capacity to function as efficient APC. Freshly isolated NK cells that express very low levels of class II HLA were also capable of presenting TT to TT-specific T cell clones, although much less efficiently. The capacity of the freshly isolated NK cells to present Ag varied considerably from donor to donor, which may be due to the variable levels of HLA-DR molecules expressed on these cells. It is possible that a certain number of activated NK cells was present in some PBMC preparations obtained from normal donors. In addition, it is still controversial whether or not all the resting NK cells do not express the class II HLA Ag (19, 22). On the other hand, several cell types, including fibroblasts, epithelial and endothelial cells, as well as human-activated T cells, can express class II HLA molecules, but their role as APC has been debated because of failure to demonstrate presentation of soluble Ag (23-25). It has been reported that activated T cells and class II HLA thyroid epithelial cells can present denatured protein Ag or peptides to stimulate proliferation of Ag-specific T cell lines (25, 26), suggesting that the defect was at the level of Ag processing. An alternative possi-

bility, supported by recent work on activated T cells by Lanzavecchia et al. (27), is that these cells are limited in their ability to present conventional Ag such as TT by their inefficiency at Ag capture. Since NK cells have the capacity to present soluble proteins such as TT or *Der p* I, this implies that they can take up Ag nonspecifically. One possibility is that CD16 (Fc γ RIII) and/or related molecules, which are absent on most T cells, are involved in capturing Ag.

It is known that cytokines released by APC exert costimulatory activity in stimulating T cell proliferation. NK cell clones produce high levels of GM-CSF, IFN- γ , and IL-3 and express mRNA for TNF- α and IL-2 (H. Spits, unpublished data). In contrast, they fail to synthesize IL-1 β and IL-6, which are considered the most important cytokines in determining accessory functions of monocytes and transformed B cells (4, 5). The fact that activated NK cells produce cytokines that can act on T cells and that are not synthesized by monocytes or B cells, notably IFN- γ and IL-3, may imply that the Ag-specific responses of T cells is different, depending on the type of APC involved.

In conclusion, this study demonstrates that NK cells can efficiently present soluble Ag to T cell clones. It is conceivable that NK cells can also function as APC in vivo, and more effectively even in situations in which local IL-2 production occurs, which results in NK cell activation and HLA expression.

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REFERENCES

1. Unanue, E. R. 1984. Antigen-presenting function of the macrophage. *Annu. Rev. Immunol.* 2:395.
2. Lanzavecchia, A. 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* 8:773.
3. Puré, E., K. Inaba, M. T. Crowley, L. Tardelli, M. D. Witmer-Pack, G. Ruberti, G. Fathman, and R. M. Steinman. 1990. Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. *J. Exp. Med.* 172:1459.
4. Aiello, F. B., D. L. Longo, R. Overton, L. Takacs, and S. K. Durum. 1990. A role for cytokines in antigen presentation: IL-1 and IL-4 induce accessory functions of antigen-presenting cells. *J. Immunol.* 144:2572.
5. O'Garra, A., G. Stapleton, V. Dhar, M. Pearce, J. Schumacher, H. Rugo, D. Barbis, A. Stall, J. Cupp, K. Moore, P. Vieira, T. Mosmann, A. Whitmore, L. Arnold, G. Houghton, and M. Howard. 1990. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Intern. Immunol.* 2:821.
6. Romani, N., S. Koide, M. Crowley, M. Witmer-Pack, A. M. Livingstone, C. G. Fathman, K. Inaba, and R. M. Steinman. 1989. Presentation of exogenous protein antigens by dendritic cells to T cell clones: intact protein is presented best by immature, epidermal Langerhans cells. *J. Exp. Med.* 169:1169.
7. Trinchieri, G. 1989. Biology of natural killer cells. In *Advances in Immunology*, Vol. 47. Academic Press, New York, p. 187.
8. Nagler, A., L. L. Lanier, S. Cwirla, and J. H. Phillips. 1989. Comparative studies of human FcRIII-positive and negative NK cells. *J. Immunol.* 143:3183.
9. Lanier, L. L., J. H. Phillips, J. Hackett, Jr., M. Tutt, and V. Kumar. 1986. Natural killer cells: definition of a cell type rather than a function. *J. Immunol.* 137:2735.
10. Young, H. A., and J. R. Ortaldo. 1987. One-signal requirement for interferon- γ production by human large granular lymphocytes. *J. Immunol.* 139:724.
11. Gascan, H., J.-F. Gauchat, M.-G. Roncarolo, H. Yssel, H. Spits, and J. E. de Vries. 1991. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by IL-4 and a signal provided by activated CD4 $^{+}$ T cell clones. *J. Exp. Med.* 173:747.
12. Roncarolo, M.-G., H. Yssel, J.-L. Touraine, R. Bacchetta, L. Gebuhrer, J. E. de Vries, and H. Spits. 1988. Antigen recognition by MHC-incompatible cells of a human mismatched chimera. *J. Exp. Med.* 168:2139.
13. Ottenhoff, T. H. M., and T. Mutis. 1990. Specific killing of cytotoxic T cells and antigen-presenting cells by CD4 $^{+}$ cytotoxic T cell clones: a novel potentially immunoregulatory T-T cell interaction. *J. Exp. Med.* 171:2011.
14. Spits, H., G. Keizer, J. Borst, C. Terhorst, A. Hekman, and J. E. de Vries. 1983. Characterization of monoclonal antibodies against cell surface molecules associated with the cytotoxic activity of natural and activated killer cells and cloned CTL lines. *Hybridoma* 2:423.
15. Quaranta, V., L. E. Walker, M. A. Pellegrino, and S. Ferrone. 1980. Purification of immunologically functional subsets of human Ia-like antigens on a monoclonal antibody (Q5/13) immunoadsorbent. *J. Immunol.* 125:1421.
16. Wibo, M., and B. Poole. 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B. *J. Cell. Biol.* 63:430.
17. Bhargava, V., and M. J. Colston. 1988. The processing and presentation of mycobacterial antigens by human monocytes. *Eur. J. Immunol.* 18:691.
18. Azuma, E., H. Yamamoto, and J. Kaplan. 1989. Use of lymphokine-activated killer cells to prevent bone marrow graft rejection and lethal graft-vs-host disease. *J. Immunol.* 143:1524.
19. Brooks, C. F., and M. Moore. 1986. Presentation of a soluble bacterial antigen and cell-surface alloantigens by large granular lymphocytes (LGL) in comparison with monocytes. *Immunology* 58:343.
20. Scala, G., P. Allavena, J. R. Ortaldo, R. B. Herberman, and J. J. Oppenheim. 1985. Subsets of human large granular lymphocytes (LGL) exhibit accessory cell functions. *J. Immunol.* 134:3049.
21. Peterson, M., and J. Miller. 1990. Invariant chain influences the immunological recognition of MHC class II molecules. *Nature (Lond.)* 345:172.
22. Perussia, B., and G. Trinchieri. 1984. Antibody 3G8, specific for the human neutrophil Fc receptor, reacts with natural killer cells. *J. Immunol.* 132:1410.
23. Shastri, N., B. Malissen, and L. Hood. 1985. Ia-transfected L-cell fibroblasts present a lysozyme peptide but not the native protein to lysozyme-specific T cells. *Proc. Natl. Acad. Sci. USA* 82:5885.
24. Lechler, R. I., M. A. Norcross, and R. N. Germain. 1985. Qualitative and quantitative studies of antigen-presenting cell function by using I-A expressing L cells. *J. Immunol.* 135:2914.
25. Gerrard, T. L., D. J. Volkman, C. H. Jurgensen, and A. S. Fauci. 1986. Activated human T cells can present denatured antigen. *Hum. Immunol.* 17:416.
26. Londei, M., J. R. Lamb, G. F. Bottazzo, and M. Feldman. 1984. Epithelial cells expressing aberrant MHC class II determinants can present antigen to cloned human T cells. *Nature (Lond.)* 312:639.
27. Lanzavecchia, A., E. Roosnek, T. Gregory, P. Berman, and S. Abrignani. 1988. T cells can present antigens such as HIV gp120 targeted to their own surface molecules. *Nature (Lond.)* 334:530.